Tetrahedron Vol. 47, No. 14/15, pp 2563-2572, 1991
Printed in Great Britain

0040-4020/91 $3 00+.00

MONOCLONAL ANTIBODIES WITH SEQUENCE SPECIFIC AFFINITY FOR A STEM - LOOP STRUCTURE IN DNA

Jean Chmielewski[1], Peter Schultz*
Department of Chemistry, University of California, Berkeley, CA 94720, U.S.A.

(*Received in USA* 12 *September* 1990)

Abstract - Monoclonal antibodies have been raised against an oligonucleotide with a stem-loop structure (**1**). Antibody 41H7 binds the oligonucleotide hapten with a dissociation constant of 2.0 x 10^{-6} M. The antibody shows sequence specificity in its binding, as it shows no detectable binding to a sequence (**12**) which differed from the hapten by only *three* bases in the loop. Additionally, a change of ten base pairs in the stem portion of the sequence (**13**) led to a four-fold reduction in the antibody affinity. These results indicate that antibody 41H7 mainly recognizes the single stranded region of hapten **1**, and that antibody recognition of the double stranded stem occurs to a lesser extent.

Introduction

The sequence specific binding of proteins to DNA is an integral part of many biological processes. Transcription events are mediated directly through the interactions of repressor proteins with operator sequences of DNA.[2] Other proteins such as restriction endonucleases and nuclear receptors also function in part by binding sequence specifically to DNA. To date no general approach exists for the creation of DNA binding molecules of any desired specificity. Oligonucleotides have been used to recognize specific double-stranded DNA sequences via triple helix and D-loop formation and the DNA binding domains of DNA regulatory proteins have also been used to target specific DNA sequences.[3] However, these strategies all suffer considerable limitations with respect to the nature and number of different sequences that can be recognized. Molecules which could recognize any desired DNA sequence would find use in the selective manipulation and modification of DNA and could also have therapeutic applications.

Antibodies represent an attractive candidate for the development of sequence specific DNA binding macromolecules. Monoclonal antibodies can be generated to a wide range of haptens, with high binding constants and specificity.[4] While antibodies have been generated to many forms of DNA much of this work has used polyclonal sera and gave results which are difficult to interpret.[5] Monoclonal antibodies to DNA have been isolated from mice with autoimmune diseases, but the DNA specificities of these antibodies are still under investigation.[6] Monoclonal antibodies with sequence and structural specificities, respectively, have been generated to DNA with Z and cruciform structures by immunization with DNA which was not conjugated to a carrier protein.[7]

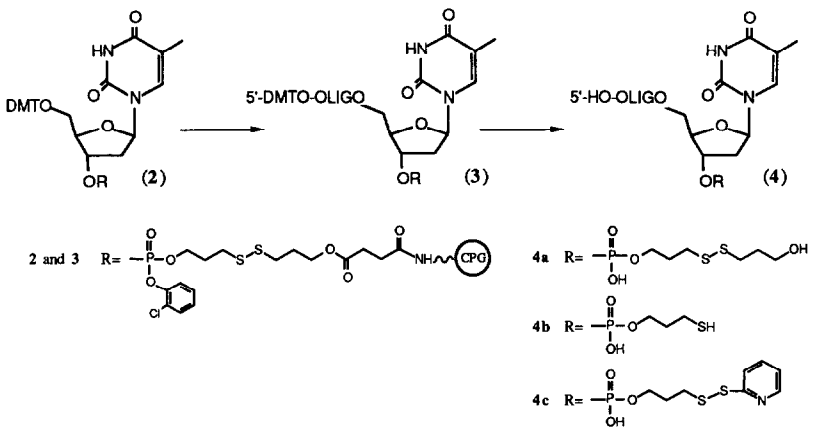
We now describe the generation and characterization of monoclonal antibodies which are capable of binding specifically to a short sequence of DNA (**1**) with a stem-loop structure.[8] Problems with dissociation of the double stranded portion of this

oligonucleotide during immunization were circumvented by incorporating the sequence into a single oligonucleotide with a stem-loop structure. In this way, the double stranded region should not denature under physiological conditions. The loop and stem sequences were chosen to avoid mismatches in base pairing of the double stranded region and so that the stem-loop structure would be the lowest energy conformation available for the oligonucleotide. Hapten **1** was conjugated to a carrier protein prior to immunization in an effort to generate a higher immune response to the oligonucleotide.

$$\begin{array}{l} \text{ACCGGCCAATTCCGGCC}\,{}^{T}_{T}\,{}^{C}_{C}\,{}^{G} \\ \text{TGGCCGGTTAAGGCCGG} \end{array} \qquad (1)$$

Preparation and Conjugation of the Hapten

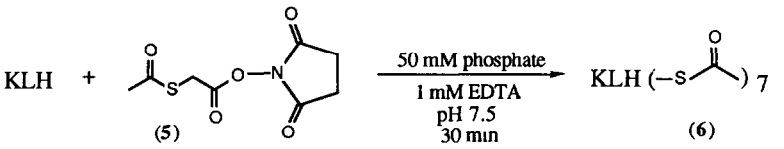
The oligonucleotide hapten **3** was prepared using a thymidine disulfide modified CPG support[9] (**2**, Scheme 1) and was synthesized using the phosphoramidite procedure on a Biosearch 8600 DNA synthesizer.[10,11] The fully protected oligonucleotide was deprotected by standard methods[11,12] and purified by reverse phase HPLC to yield **4a**. The derivatized oligonucleotide **4a** was reduced with an excess of dithiothreitol for 20 hours at 37°C in 170 mM phosphate buffer, pH 8.0 to give the free thiol **4b**, followed by gel filtration on G-50 Sephadex and direct treatment with dithiodipyridine in a pH 5.5, 100 mM phosphate buffer. Excess dithiodipyridine was removed on a G-50 size exclusion column to yield the mixed disulfide **4c**.



Scheme 1.

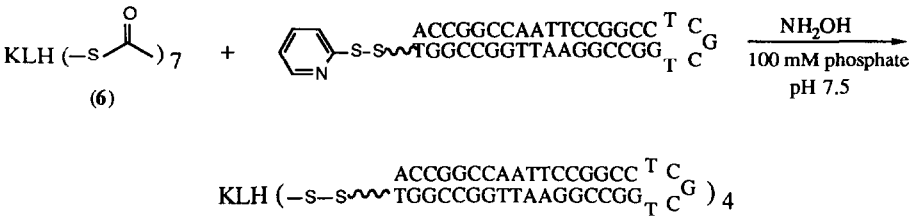
Conjugation of the hapten **4c** to the carrier proteins keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) necessitated the introduction of surface thiols into the proteins. This was accomplished by reacting the carrier protein (Scheme 2) with 30 equivalents of S-acetylthioglycolic acid N-hydroxysuccinimide ester (**5**) in a 50 mM phosphate/1 mM EDTA buffer at pH 7.5 for 30 min.[13] The proteins were dialyzed

extensively and the amount of acetylated thiol incorporated into the protein structure was determined by 2-thiopyridone formation, as measured at 343 nm, upon treatment with hydroxylamine and dithiodipyridine. The amount of 2-thiopyridone released was correlated to the number of free thiols on the surface of the protein.[14] In such a way seven and three sulfhydryl groups were incorporated into KLH and BSA, respectively.



Scheme 2.

Oligonucleotide **4c** was conjugated to thioacetylated KLH (**6**) via disulfide linkages (Scheme 3) by combining **6** and **4c** in a one to seven ratio in a 100 mM phosphate, pH 7.5 buffer containing hydroxylamine. After 4 hrs at room temperature the reaction was 68% complete as judged by thiopyridone release. After extensive dialysis against phosphate-buffered saline (PBS buffer), the epitope density for KLH was four and for BSA was two as judged by Bradford[15] and Lowry[16] tests.



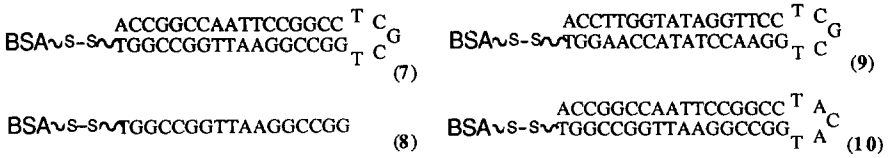
Scheme 3.

Antibody Production and Purification

NZB mice were immunized with the KLH conjugate of **4c** and titers were measured with the BSA conjugate of **4c** in an enzyme-linked immunosorbent assay (ELISA)[17] using mouse IgG (whole chain). After 4 injections, monoclonal antibodies were produced following a standard protocol.[18] Ascites fluid from IgM 41H7 was dialyzed against 2X PBS, precipitated with a 10 mM Tris buffer (pH 7.5) and purified to homogeneity on a G-200 Sephadex column (2X PBS) as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis.[19] To circumvent the lack of solubility of IgM antibodies in low salt buffers, we generated the highly soluble monomer form of the IgM.[20] IgM 41H7 was treated with 10 mM dithiothreitol in a 200 mM Tris pH 8.6 buffer for 1 hr at room temperature, followed by alkylation with 11 mM iodoacetamide at pH 8.0 for 15 min at room temperature. After extensive dialysis, the reduced-alkylated monoclonal antibody was purified to homogeneity on an S-300 Sepharose column with PBS buffer.

Binding Selectivities

To test the binding selectivity of the reduced-alkylated IgM 41H7 for DNA sequences we generated oligonucleotides with different sequences (**8-10**). The oligonucleotide in **10** has a different loop sequence from the hapten, whereas **9** has a different stem sequence. By using these sequences, cell lines producing antibodies could be screened for specificity to the stem or loop sequences of the hapten or structural specificity to the stem-loop. The cell lines could also be screened for affinity to a single strand corresponding to the stem of the hapten by using **8**. These oligonucleotide sequences were conjugated to BSA via the disulfide methodology described earlier, giving epitope densities for conjugates **7-10** between seven and nine as measured by Bradford[15] and Lowry[16] tests.



We used an enzyme-linked immunosorbent assay (ELISA)[17] with alkaline phosphatase conjugated to mouse IgG (whole molecule) in our initial screening of cell lines for sequence-specific binding affinities. By coating the ELISA plate with equivalent amounts of the BSA conjugates, the absorbance at 405 nm, obtained from hydrolysis of nitrophenyl phosphate, can be correlated to relative binding affinities of the antibody for that DNA sequence (Table 1). IgM 41H7 showed a sequence dependent binding of the hapten, binding the mutated sequences with reduced affinity or affinities which were indistinguishable from background. Seven other monoclonal antibodies were generated from this procedure and each showed the same affinity for oligonucleotide sequences **7-10** and thioacetylated BSA in an ELISA assay.

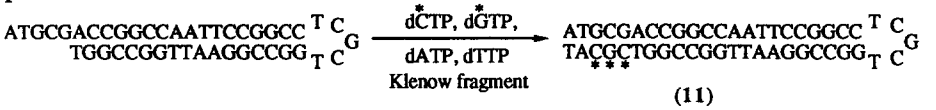
Table 1. Relative Binding Affinities of 41H7 via ELISA Assay

Oligonucleotide	Absorbance*	Relative value
7	1.12	1.0
8	0.162	0.14
9	0.376	0.36
10	0.172	0.15
background**	0.152	0.14

*Due to hydrolysis of nitrophenyl phosphate by alkaline phosphate coupled to mouse IgG (whole chain)
** No BSA conjugate was coated on the ELISA plate

Binding constants were obtained for the interaction of monoclonal antibody 41H7 with the three stem-loop sequences using α-^{32}P-labelled oligonucleotides in a gel shift assay.[21] Three oligonucleotides containing a 5'-five base overhang were synthesized

using the standard phosphoramidite procedure on controlled pore glass[11] and purified on a 15% polyacrylamide gel. The recessed end was filled in using α-^{32}P-radiolabelled deoxy-CTP and deoxy-GTP, and unlabelled deoxy-ATP and deoxy-TTP using the Klenow fragment (large fragment) of *E. coli* DNA polymerase I (Scheme 4) in a 10 mM Tris/6 mM $MgCl_2$/1 mM dithiothreitol, pH 8.0 buffer at room temperature for 30 min.[22] The α-^{32}P labelled oligonucleotides sequences **11-13** were gel purified on a 15% polyacrylamide gel, extracted from the gel, desalted and used directly in the binding experiments.



(11)

(12)

(13)

Scheme 4.

The binding affinities of the oligonucleotides **11-13** for reduced-alkylated IgM 41H7 were determined using a gel shift assay.[21] By keeping the DNA concentration constant and much lower than the protein concentrations, and varying the protein concentration, the half maximal point in the binding curve (or the point in which half of the DNA is unbound) can be correlated directly to the dissociation constant. In our experiments the second band on the gel, corresponding to the bound DNA species, was too diffuse to be seen (Figure 1), therefore we used the amount of unbound DNA to determine the dissociation constants. By measuring the antibody concentration at the point in which half of the labelled DNA is in the unbound state, we were able to determine the dissociation constants for the interaction of IgM 41H7 and the three oligonucleotides (Table 2).

Table 2. Dissociation Constants for Monoclonal Antibody 41H7 and Oligonucleotides

11	2.0×10^{-6} M
12**	$> 2.5 \times 10^{-5}$ M
13	7.2×10^{-6} M

**No binding was detected up to an [Ab] of 2.5×10^{-5} M

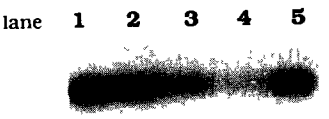


Figure 1. Gel shift assay showing the decrease in the amount of free DNA with increasing [Ab]. [Ab]: lane 1, 4.2×10^{-7} M; lane 2, 8.5×10^{-7} M; lane 3, 4.2×10^{-6} M; lane 4, 8.5×10^{-6} M; lane 5, no Ab.

A monoclonal antibody which binds a stem-loop structure of DNA has been generated. Antibody 41H7 also shows sequence specificity in its binding as it shows no

detectable binding to sequence **12** which differed from the hapten by only *three* bases. Additionally, by keeping the loop sequence intact and changing a portion of the internal sequence of the stem portion as in **13**, the binding affinity is reduced by a factor of about four. Similar results are obtained with both the ELISA and gel shift assays. These experiments demonstrate that the monoclonal antibody is making sequence specific contacts with the loop portion of the oligonucleotide as its major interaction. It seems that there are some weaker contacts being made with a portion of the stem structure also, since a change of ten base pairs in the stem leads to a drop in the binding affinity. This result might also be accounted for by a change in the overall structure of the stem-loop, due to the sequence change, which would affect the conformation of the loop sequence.

These results suggest that it is possible to raise antibodies with sequence specificity for DNA. Interestingly, the single stranded loop appears to be more immunodominant than the stem region for hapten **1**, suggesting that antibodies may best serve as single stranded nucleic acid or conformational probes. The ability to generate monoclonal antibodies which bind sequence specifically to a DNA sequence has potential for generating repressor-like molecules to any sequence of DNA. With the ability to incorporate DNA cleaving reagents on to the surface of proteins[23], we have the possibility of generating new classes of sequence specific restriction enzymes using monoclonal antibody technology.

Experimental

Oligonucleotide **4a**

The support bound oligonucleotide **3** (30 mg, ~1μmol) was treated with 80% pyridine in water (1 ml) containing 2-pyridine aldoxime (76 mg, 0.62 mmol) and tetramethylguanidine (78 μl, 0.62 mmol), at 37°C for 20 hrs. To this mixture was added concentrated NH_4OH (6 ml) and the reaction was kept in a tightly sealed vial for 5 hrs at 55°C. The solvent was removed under reduced pressure to a volume of 500 μl, and the crude 5'-DMT oligonucleotide was desalted on a C18 Sep-Pak (Waters) with 10 ml of 25 mM TEAB (pH 7.6) used as the loading buffer, and 5 ml of 30% CH_3CN, 50 mM TEAB (pH, 7.6) used for the elution buffer. The desalted solution was concentrated to 1.5 ml and purified on reverse phase HPLC.[9] The purified material was concentrated to dryness and 1 ml of 80% HOAc was added. After 30 min the solution was concentrated to near dryness under reduced pressure, 250 μl of 500 mM Tris, 2 mM EDTA, pH 8.0 was added and the mixture was washed with ether (4 x 250 μl) to yield oligonucleotide **4a**.

Oligonucleotide **4c**

The detritylated oligonucleotide **4a** was treated with a solution of dithiothreitol (5 mg, 0.032 mmol) in 500 μl of H_2O, and the resulting mixture was stored at 37°C for 20 hrs. The free thiol **4b** was purified on a G-50 Sephadex size exclusion column (50 mM Tris, 0.2 mM EDTA, pH 8.0 buffer), and the fractions corresponding to **4b** were

collected directly into a solution of dithiodipyridine (2.0 mg per 10 ml fraction) in 2 ml of a 100 mM phosphate buffer pH 5.5, containing 15% acetonitrile. This mixture was allowed to react overnight, concentrated under reduced pressure, and purified on a G-50 Sephadex column (50 mM Tris, 0.2 mM EDTA, pH 8.0 buffer) to yield oligonucleotide **4c**. The yield of **4c**, starting from **2**, based on the OD_{260} was 2.7 mg (23%, OD_{260}/OD_{280}=1.60). The yield based on the absorbance of the thiopyridyl anion at 343 nm (ε=7060 M^{-1}) after cleavage with 10 mM DTT was 3.1 mg (29%).

Thioacetylated KLH (**6**)

KLH (10 mg, 0.14 μmol) was dissolved in 2 ml of 50 mM phosphate, 1 mM EDTA, pH 7.5. S-acetylthioglycolic acid N-hydroxysuccinimide (**5**) (0.5 mg, 2.2 μmol) in 20 μl of DMF was added to the KLH solution and the mixture was stirred for 30 min at room temperature. After derivatization the protein was dialyzed extensively against 50 mM phosphate, 1 mM EDTA, pH 7.0 to yield the thioacetylated KLH **6**. The acetyl groups were removed with a solution of hydroxylamine hydrochloride (500 mM) in a approximately 100 mM phosphate, pH 7.5 buffer, and the sulfhydryl content was measured with dithiodipyridine.

KLH Conjugate of **4c**

Thioacetylated KLH **6** (5.8 mg, 2.6 mg/ml) in a 100 mM phosphate, 1 mM EDTA, pH 7.0 buffer and oligonucleotide **4c** (1.9 ml, 0.185 mM) in a 200 mM Tris, 1 mM EDTA, pH 8.0 buffer were combined with hydroxylamine hydrochloride (415 μl, 500 mM) in a 100 mM phosphate, 2 mM EDTA, pH 7.5 buffer. After 1.5 hrs at room temperature the reaction was 50% complete as judged by thiopyridone release at 343 nm. Additional **6** (200 ul, 2.6 mg/ml) was added and the reaction went to 68% in another 2.5 hrs. The reaction was dialyzed extensively with PBS buffer (3 x 1.5 l) and the epitope density was determined to be 3.6 as judged by Lowry[15] and Bradford[16] assays.

Reduced-Alkylated IgM 41H7

Monoclonal antibody 41H7 (5 mg, 2 ml) was dialyzed against a 200 mM Tris, pH 8.6 buffer. This solution was treated with a solution of DTT (220 ml, 100 mM) in 200 mM Tris, pH 8.6 for 1 hr at room temperature. The mixture was diluted 1:1 with 200 mM Tris, pH 7.3, followed by treatment with a solution of iodoacetamide (240 ml, 110 mM) in H_2O for 15 min at room temperature. The mixture was dialyzed extensively against PBS and purified on a S-300 Sepharose column (PBS, 10 ml fractions). Fraction 24-27 containing reduced-alkylated 41H7 were pooled, concentrated by vacuum dialysis and judged to be homogeneous by SDS-PAGE.

ELISA Binding Assay

Solutions of BSA conjugates **7**-**10** (1 μg, 100 μl) in 100 mM Tris, 150 mM NaCl, pH 7.4 buffer were added to each well of a 96 well ELISA plate (Immulon II) and incubated for 12 hrs at 4°C. The plate was washed with N4 buffer (150 mM NaCl, 50 mM Tris, 20 μM $ZnCl_2$, 1 mM $MgCl_2$, and 0.03% sodium azide, pH 7.4) and 100 μl of

the supernatant of the cell culture 41H7 was added to each well. After incubation for 1 hr at room temperature, the plate was washed with N4 buffer and a solution of alkaline phosphatase conjugated to mouse IgG (whole chain specific) (0.1 μg, 100 μl) in N5 buffer (N4 buffer with 0.25% gelatin) with 10% calf serum was added to each well. After incubation for 4 hrs at room temperature, the wells were washed with N4 buffer and dried thoroughly. A solution of p-nitrophenyl phosphate (100 μg, 100 μl) in DEA buffer (1 M diethanolamine, 500 μM $MgCl_2$, pH 9.8) was added to each well and the absorbance produced by *p*-nitrophenoxide at 405 nm was measured after 30 min with a SLT-Labinstruments EAR 400AT plate reader. Duplicate runs were performed on each plate and the values are shown in Table 1.

Radiolabelling of Oligonucleotides

To a solution of each oligonucleotide **11-13** (7 ng, 0.53 pmol) in H_2O (2 μl) at 0°C was added H_2O (2.5 μl), 10X buffer (2.5 μl; 100 mM Tris, 60 mM $MgCl_2$, 10 mM DTT, pH 8.0), dNTP (1 μl, 2 mM solution), α-^{32}P-dCTP (5 μl, 10 μCi/μl)), α-^{32}P-dGTP (5 μl, 10 μCi/μl) and Klenow fragment (1 μl, 5 units/μl). The mixture was incubated for 30 min at room temperature and heated to 70°C for 5 min. Gel loading buffer (8 μl, 30% glycerol in H_2O, 0.25% bromophenyl blue, 0.25% xylene cyanol) was added, the mixture was heated to 70°C for 2 min, and 15 μl of the reaction was loaded into each of the two wells of a 15% denaturing polyacrylamide gel. The gel was run at constant power (10 watts) until the xylene cyanol dye ran off the bottom of the gel. The desired bands were cut from the gel and extracted with 2 x 500 μl of extracting buffer (100 mM sodium acetate, 10 mM magnesium acetate, pH 6.5). After phenol/$CHCl_3$ extractions, the solutions of oligonucleotides were desalted on a Nap5 column (Biorad) and lyopholized to dryness. The labelled oligonucleotides were diluted with 530 μl of H_2O to a concentration of 1 x 10^{-9} M and used directly in the gel shift assays.

General Protocol for Gel Shift Assays

To each binding experiment was added an aqueous solution of labelled oligonucleotide **11-13** (1.5 μl, 1 x 10^{-9} M), glycerol (1.5 μl), appropriately diluted reduced-alkylated IgM 41H7 (5 μl) in a 30 mM Pipes, pH 7.0 buffer, and H_2O (7 μl). The final DNA concentration was 1 x 10^{-10} M and the final antibody concentrations were between 2.5 x 10^{-5} M and 8.5 x 10^{-7} M. The reactions were equilibrated at room temperature for 30 min and 10 μl were loaded directly on to a pre-electrophoresed, 15% non-denaturing gel with a recirculating 10 mM Pipes, pH 7.0 running buffer. While loading the samples the gel was run at a constant voltage of 300 V, which was lowered to 100 V when the samples had run into the gel. After approximately 4 hrs at 100 V, the gel was exposed for 5-10 hrs with an intensifying screen to give the data shown in Figure 1. The dissociation constant was obtained from the antibody concentration at the point in which half of the DNA was still in the unbound state.

Acknowledgements

We thank the National Institutes of Health (Grant R01 AI24695 to P. S. and Postdoctoral Fellowship F32 GM 12065-02 to J. C.) for their support of this work. We would also like to acknowledge Jim Prudent for providing technical advice and assistance with antibody production, Dr. Ron Zuckermann and Dehua Pei for providing the modified CPG support, and Dan Suich for helpful advice with the gel shift assay.

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8. Abbreviations used in text: CPG, controlled pore glass; KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; NTP, nucleotide triphosphate; TEAB, triethylammonium bicarbonate buffer; DEA, diethylamine; SDS PAGE, sodiun dodecyl sulfate polyacrylamide gel electrophoresis.
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