MOLECULAR MODELING AND DOCKING ANALYSIS OF THE VARIABLE REGIONS OF AN ANTI-N6-METHYLADENOSINE MONOCLONAL ANTIBODY

Avni Patrick Nimani
Northern Michigan University

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MOLECULAR MODELING AND DOCKING ANALYSIS OF THE VARIABLE REGIONS OF AN ANTI-N\textsuperscript{6}-METHYLADENOSINE MONOCLONAL ANTIBODY

By

Avni Patrick Nimani

THESIS

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SIGNATURE APPROVAL FORM

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ABSTRACT

MOLECULAR MODELING AND DOCKING ANALYSIS OF THE VARIABLE REGIONS OF AN ANTI-N\(^6\)-METHYLADENOSINE MONOCLONAL ANTIBODY

By

Avni Patrick Nimani

The previously unknown DNA and amino acid sequences of the 7C7:C5 anti-N\(^6\)-methyladenosine (\(6^m\)A\(_{os}\)) variable fragment (Fv) are reported herein. The 7C7:C5 Fv was molecular modeled using a novel approach involving alignment of numerous high identity antigen-bound crystallographic structures and antigen-unbound crystallographic structures as templates, in which a binding pocket was revealed only in the antigen-bound-model of the Fv. Rigid Fv/flexible antigen docking and flexible Fv/flexible antigen docking of deoxy-N\(^6\)-methyladenosine (d\(6^m\)A), deoxyadenosine (dA), and single-stranded (ss) DNA trinucleotide (A-\(6^m\)A-T) revealed three critical residues (TYR L48, SER L55, and PRO H103) to be involved in docking of d\(6^m\)A and related antigens to the 7C7:C5 Fv. The N6 methyl group in d\(6^m\)A was predicted to make more van der Waals contacts than the corresponding N6 hydrogen in dA leading to an estimated 6 to 10 fold greater affinity for d\(6^m\)A than dA. Intermediate dissociation constant (K\(_d\)) calculations suggest a K\(_d\) range of 7.835 x 10\(^{-7}\) M to 5.92 x 10\(^{-8}\) M for the binding of d\(6^m\)A in ssDNA, which corresponds to an affinity most appropriately used for genotyping or diagnostics. The binding interactions suggest the possibility of a slightly higher affinity for ribonucleoside versus deoxyribonucleoside, suggesting that the 7C7:C5 Fv may be able to bind to N\(^6\)-methyladenosine (\(6^m\)A\(_{os}\)) in RNA.
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2009
ACKNOWLEDGMENTS

I would like to thank Dr. Mark Paulsen, my thesis advisor, Dr. Osvaldo Lopez, one of my thesis committee members, and Dr. Lesley Putman, one of my thesis committee members. Dr. Paulsen and Dr. Putman contributed to the project through their knowledge of protein and DNA structures. Dr. Paulsen also contributed his knowledge on computer modeling and protein-DNA interactions. Dr. Lopez provided the 7C7:C5 MAb and contributed his knowledge on antibodies, molecular biology, and immunochemistry in relation to the 7C7:C5 MAb and methylated DNA. I would like to thank Frederico J. Hoffman for his previous work with the characterization of the 7C7:C5 MAb, which helped to make the current study possible. Additionally, I would like to thank Dr. Robert Lyons, the University of Michigan DNA Sequencing Core director, and his lab technicians for sequencing the 7C7:C5 plasmid samples. Lastly, I would like to thank Pauline Gould for assisting with scheduling and arrangements regarding this thesis. My thesis was reviewed and critiqued by Dr. Paulsen and other members of my thesis committee to ensure that it is of the highest quality.

Initially funding was provided by Dr. Terry Seethoff, dean of the College of Arts and Sciences. Additional funding for the project was obtained through an Excellence in Education Grant and a Charles C. Spooner Grant. Dr. Osvaldo Lopez provided general laboratory equipment and reagents and funding for EcoRI methylase. Dr. Mark Paulsen provided funding for additional Taq polymerase. The format of references prescribed by *Nature* 2009 and the Department of Chemistry is used herein.
PREFACE

In my first year (2003) at Northern Michigan University as a Master of Science student, I primarily focused on classes and had some early discussions about the kind of research I would like to perform with my Academic Advisor, Dr. Mark Paulsen. At the time, I was interested in working with computer modeling. During the start of my second year (Fall 2004), I opted to take a course in Virology instructed by Dr. Osvaldo Lopez. After speaking with Dr. Osvaldo Lopez for some time during this course, a project working with the 7C7:C5 monoclonal antibody was arrived at. Also, in Fall 2004, I took a course in Protein Structure and Function with Dr. Lesley Putman, which helped me to better understand protein and nucleic acid structure in relation to function. In this class, I obtained hands-on experience with analyzing three-dimensional molecular models. Early lab work in 2004 involved becoming familiar with molecular biology techniques such as plasmid isolation that would prove useful towards completion of the project. The earliest work reported herein involved blotting on membranes for a general characterization of the specificity of the 7C7:C5 monoclonal antibody.

Early in 2005, the project took shape towards obtaining a three-dimensional molecular model of the 7C7:C5 variable fragment. The initial stages of this project required obtaining the DNA sequences for the 7C7:C5 variable regions. Acquiring funding for laboratory work towards obtaining the DNA sequences for the variable regions of the 7C7:C5 monoclonal antibody was a major obstacle in the following year (2005) and, thus, much effort was placed towards acquiring enough funding. In Fall 2006, I enrolled in a molecular modeling course with Dr. M. Paulsen, which helped me to
understand and appreciate the theory behind computational modeling. During this course, I also became more familiar with general use of computational modeling. In Winter 2005, I enrolled in a Biotechnology course with Dr. O. Lopez, which focused on antibody development and modern day uses of antibodies. Upon obtaining funding in 2006, much laboratory work was performed over a two year period, in which the DNA sequences for the 7C7:C5 variable regions were successfully sequenced. In 2008, molecular modeling and docking analysis of the 7C7:C5 variable fragment was performed leading me to the end of my research at Northern Michigan University.

My research at NMU was time-consuming but quite enjoyable and rewarding. I have thoroughly benefited from all the techniques I have learned and all the skills I have gained. I honestly feel I have completed a worthwhile project and I am glad that my time and diligent effort have been spent on such a project. For the idea behind the project and their help towards the project, I have to sincerely thank Dr. O. Lopez and Dr. M. Paulsen. Also, I would like to thank them for their help with obtaining funding for the project. They have spent much time and effort in guiding me through completion of my thesis work and their efforts are much appreciated. Lastly, I would like to thank all three members of my thesis committee, Dr. M. Paulsen, Dr. O. Lopez, and Dr. L. Putman, for their assistance regarding my thesis.
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Symbols and Abbreviations

A-6mA-T……………….adenosine-N⁶-methyladenosine-thymidine trinucleotide
ADT……………………AutoDockTools
amIgG-AP………………anti-mouse IgG conjugated to alkaline phosphatase
APH(3’)-IIIa…………….aminoglycoside phosphotransferase enzyme
6mA……………………N⁶-methyladenine
6mA₆s……………………N⁶-methyladenosine
bp…………………….base pair
cDNA………………….copy DNA
CDR…………………….complementarity-determining region
C₃H1…………………….constant region 1 of heavy chain
C₃κ…………………….constant region of kappa light chain
CV NY-2A………………Chlorella virus NY-2A
5mC…………………….5-methylcytosine
5mC₆s…………………….5-methylcytidine
dA…………………….deoxyadenosine
d6mA…………………….deoxy-N⁶-methyladenosine
d6mATP…………………deoxy-N⁶-methyladenosine triphosphate
d5mC…………………….deoxy-5-methylcytidine
DNA…………………….deoxyribonucleic acid
ds……………………..double-stranded (in regards to DNA)
dT………………….deoxythymidine
dT₃………………….deoxythymidine trinucleotide
dT₅..........................deoxythymidine pentanucleotide

E.coli........................Escherichia coli

ELISA........................enzyme-linked immunoassay

EtBr..........................ethidium bromide

ExPASy.........................Expert Protein Analysis System

Fab............................antigen-binding fragment

Fc..............................crystallizable fragment

FD................................FastDigest

FDU.........................FastDigest units (associated with protein amount)

FR..............................framework region (of the variable region)

Fv..............................variable fragment

gp..............................glycoprotein

IgG..............................immunoglobulin G or immunoglobulin gamma

Kₐ..............................association constant

K₈..............................dissociation constant

LB..............................Luria-Bertani

LBA..........................LB with ampicillin

L-BFGS..........................limited memory Broyden-Fletcher-Goldfarb-Shanno

Lₕ............................leader sequence of the heavy chain

Lₖ............................leader sequence of the kappa light chain

MAb..........................monoclonal antibody

M.EcoRI.......................EcoRI methyltransferase

mRNA..........................messenger RNA
MT..........................methyltransferase or methylase
M.TaqI........................TaqI methyltransferase
NCBI..........................National Center for Biotechnology Information
PAb..........................polyclonal antibody
PBS..........................phosphate buffered saline
PCR............................polymerase chain reaction
PDB..........................Protein Data Bank
pdfs..........................probability density functions
RE............................restriction endonuclease
RMSD..........................root mean square deviation
RNA............................ribonucleic acid
RT............................reverse transcription
SAM...........................S-adenosyl-L-methionine
sc.............................single-chain (in regards to Fv)
siRNA..........................small interfering RNA
ss..............................single-stranded (in regards to DNA)
U.................................units (associated with protein amount)
UV..............................ultraviolet
V.................................variable (associated with region)
V_H..............................variable region of heavy chain
V_κ..............................variable region of kappa light chain
INTRODUCTION

DNA Methylation

DNA methylation is an ordinary biological process in which a methyl group (—CH₃) or methyl groups covalently link(s) to DNA. Methylation of DNA is distinct from other chemical modifications of DNA because methylation is a natural process of altering genes and does not result in damaging the DNA reading frame like alkylation, deamination, or oxidative damage. In prokaryotes, DNA methylation acts as a defense against bacteriophages, aids in DNA repair, and regulates DNA replication. In eukaryotes, DNA methylation is a method of regulating gene expression, inactivating mammalian X-chromosomes, and protecting DNA.

For decades, DNA methylation has been known to produce the following methylated bases: N⁶-methyladenine (⁶mA), N⁴-methylcytosine (⁴mC), and 5-methylcytosine (⁵mC). The nucleotide sequences listed in Table 1 refer to five prokaryotic, naturally occurring sites of methylation for N⁶ of adenine. In addition to prokaryotes, DNA methylation of adenine has been found to naturally occur in “higher plants,” of which an adenine methylase known as wadmntase was isolated from wheat seedlings (eukaryotes). The presence of ⁶mA has been found in the DNA of various invertebrates (mosquitoes, fruit flies, and mealybugs), particularly in association with gravidity or embryonic development. The presence of ⁶mA has also been found in rat, bovine, and human sperm DNA, although one study contradicts those findings. Furthermore, the presence of ⁶mA was observed in a steroid reductase gene expressed in the testis and epididymis of adult rat. As a result, although ⁶mA is most commonly found to
naturally occur in prokaryotes, it also appears to be present in higher plant and animal genes associated with reproduction.

Table 1. Methylases and their sites of methylation

<table>
<thead>
<tr>
<th>Methylases</th>
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<tr>
<td>M.EcoRI</td>
<td>GA&lt;sup&gt;6m&lt;/sup&gt;ATTC</td>
</tr>
<tr>
<td>M.dam</td>
<td>G&lt;sup&gt;6m&lt;/sup&gt;ATC</td>
</tr>
<tr>
<td>M.TaqI</td>
<td>TCG&lt;sup&gt;6m&lt;/sup&gt;A</td>
</tr>
<tr>
<td>M.BcgI</td>
<td>CG&lt;sup&gt;6m&lt;/sup&gt;ANNNNNNTGC</td>
</tr>
<tr>
<td>M.AloI</td>
<td>GA&lt;sup&gt;6m&lt;/sup&gt;ACNNNNNNTCC</td>
</tr>
</tbody>
</table>

*N = any nucleotide

Methyltransferases/methylases (MTs) require S-adenosyl-L-methionine (SAM), as a cofactor which contributes the methyl group to be added to a specific nucleotide base of a restriction site sequence<sup>1</sup>. The catalytic mechanism is believed to involve direct transfer of the methyl group from SAM to the nucleotide base via <sup>SN</sup>2 reaction<sup>15-17</sup>. The reaction requires coordinated binding of SAM and the target base for methylation. In the case of M.TaqI, the crystal structure revealed a cleft of 15 Å between the methyl group of SAM and the N6 of the target adenine, which would normally provide quite an obstacle for direct transfer of a methyl group<sup>18</sup>. As a result, base-flipping, or distortion of a base out of its usual position in the double helix, is believed to occur to bring the methyl group of SAM in closer proximity to the N6 of the target adenine<sup>18-21</sup>.

Cytosine and adenine binding in MTs are quite distinct. While both cytosine MTs and adenine MTs have arginine residues that are positioned for electrostatic interactions between the DNA binding cleft and the DNA backbone, the two types of DNA MTs differ in their binding interactions with their target bases<sup>1,18,22</sup>. Hydrogen bonding interactions are believed to play a more significant role in cytosine binding in MTs, while hydrophobic interactions are structurally expected to predominate in adenine binding in MTs<sup>22</sup>.
In the case of adenine MTs, hydrophobic residues particularly phenylalanine, tyrosine, isoleucine, leucine, and valine in the SAM binding region and in the target adenine region have been structurally suggested in making favorable face-to-face or edge-to-face van der Waals contacts with the adenine moiety in SAM and the target adenine in DNA, respectively. In addition, positively charged residues have been found to interact with phosphate groups of the DNA backbone and have also been implicated in direct interaction with the target base for methylation, while negatively charged residues have been found to surround the sulfur group of SAM within a radius of 4-6 Å and to be involved in hydrogen bonding and van der Waals contacts with the adenine moiety of SAM. These interactions are likely to be important for the binding of adenine in MTs and potentially other proteins.

DNA genotyping methods predominantly use restriction endonucleases (RE)s; however, MTs can also be used in genotyping. In fact, the use of MTs offers advantages in DNA mapping. The most significant advantage is that site specific methylation can be applied to PCR-based formats which are more rapid than RE formats based on restriction fragment length polymorphism (RFLP) genotyping, the standard genotyping method from 1985 until the early 1990’s. Methylation based PCR genotyping formats require amplification of DNA, DNA fragmentation, site specific methylation, DNA denaturation, DNA fixation, and immunochemical detection, while RFLP genotyping requires probe labeling, DNA fragmentation, gel electrophoresis, membrane transfer blotting, hybridization of probe with target DNA, membrane washing, and autoradiography. As a result, another advantage is that using DNA MTs in combination with REs allows for the DNA to be mapped without the use of radiometric
labeling and gel electrophoresis. A final advantage is the ability of some MTs to recognize short sequences—2 to 3 base pairs in length.

**Antibodies**

Antibodies have the ability to be produced against any known molecule and, thus, antibodies against DNA can be used as a means of detecting DNA in genotyping methods. Antibodies are tetrameric proteins produced by B cells as part of the immune response against foreign molecules and organisms that function to neutralize foreign molecules. Antibodies are composed of two identical heavy chains (~125 kDa for both) and two identical light chains (~25 kDa for both) and have a total molecular weight of ~150 kDa.

The two heavy chains are covalently linked to one another through disulfide bonds, in a domain known as the hinge region. Due to the limited number of disulfide bonds, noncovalent interactions such as hydrogen bonds, electrostatic interactions, van der Waals interactions, hydrophobic interactions, and C_H2 domain carbohydrate-carbohydrate interactions are also important for the association of the two heavy chains with one another. Each heavy chain is covalently linked to a light chain via a disulfide bond. In addition to disulfide bonds, the heavy chains associate with the light chains through noncovalent interactions. Each heavy chain has five domains and each light chain has two domains. In each chain, disulfide bonds have also been found to covalently link one end of a domain to the other end of the same domain, with the exception of the hinge region.
Together the heavy chains and light chains are comprised of two main regions: the constant (C) region and the variable (V) region. Various antibody heavy chain isotypes naturally occur in the immune system: immunoglobulin mu (IgM), gamma (IgG), alpha (IgA), delta (IgD), and epsilon (IgE). These isotypes are classified according to the conformation and properties of the constant regions. The antibody in the current study is an immunoglobulin G (IgG) isotype. Antibodies of this isotype are the smallest yet most abundant of antibodies (75-80% of all antibodies) and are present in all bodily fluids. Immunoglobulin Gs are typically produced in high quantity as part of the secondary antibody response.

Each heavy chain of an IgG antibody is composed of three constant domains (CH1, CH2, and CH3), a hinge region, and a variable domain (VH). Amino acid sequences for the VH are generally around 120 amino acids long. The light chain of an IgG is composed of a constant domain (CL) and a variable domain (VL). Two subclasses of light chains occur in IgGs: a kappa light chain and a lambda light chain. The kappa light chain occurs more frequently than the lambda counterpart in IgGs in serum. Amino acid sequences for the variable kappa region (Vk) are typically around 110 amino acids long.

The variable domain of each chain (light or heavy) is comprised of four framework regions (FRs)—FR-(L/H)1, FR-(L/H)2, FR-(L/H)3, and FR-(L/H)4—and three hypervariable regions called complementarity determining regions (CDRs): CDR-(L/H)1, CDR-(L/H)2, and CDR-(L/H)3. The CDRs contain the majority of residues that contact an antigen and typically either partially or completely form loops. The most variable of the CDRs is CDR-H3. Murine IgG antibodies
have CDR-H3s that range from 2-19 residues in length and human IgG antibodies have CDR-H3s that range from 2-26 residues in length. Crystal structures of Fab-oligonucleotide complexes have shown that the length of the CDR-H3s are typically directly related to the length of the segments of epitopes (sites on an antigen that interact with residues of the antibody variable region that directly involve binding of the antigen to the antibody) that interact with them.

Limited papain digestion has been used to separate IgG antibodies into three main functional fragments: a crystallizable fragment (Fc) and two antigen-binding fragments (Fabs). The Fc is comprised of a pair of C\textsubscript{H}2 and C\textsubscript{H}3 domains and interacts with Fc\textsubscript{γ} receptors on macrophages and other cells and effector molecules involved in molecular processes that particularly aid in destruction of pathogens. Each Fab contains a dominant or determining fragment (Fd), which is comprised of the C\textsubscript{H}1 domain and the V\textsubscript{H}, linked to a light chain, which is comprised of the C\textsubscript{L} and the V\textsubscript{L}. The minimal antigen-binding fragment or variable fragment is the Fv. Each IgG antibody has two Fv, one from each Fab. Each Fv contains a V\textsubscript{H} and a V\textsubscript{L} associated together through noncovalent interactions. Each Fv has a total of six CDRs (three from the V\textsubscript{L} and three from the V\textsubscript{H}). Figure 1 illustrates the general composite of an IgG antibody.
Anti-Nucleic Acid Antibodies

Antibodies bind to proteins, nucleic acids, or other antigens. Antibodies of the same subclass have highly conserved constant regions and attain most of their uniqueness in binding specificity through distinct variable regions. Polyclonal antibodies (PAbs) are antibodies with different variable regions produced against an antigen. Monoclonal antibodies (MAbs) are antibodies of the same class with identical variable regions produced against an antigen. Polyclonal antibodies are less specific to a particular antigen than MAbs due to the presence of many different variable regions. Some of these variable regions may detect other antigens occasionally giving false positives in detection of a specific antigen. Thus, MAbs are preferred in detection of antigens because they are produced selectively to be specific for a particular antigen. More specifically, MAbs produced against an antigen bind to residues in a single distinct region of the antigen known as the epitope, while PAbs produced against an antigen may bind to different epitopes on the same antigen.
Monoclonal antibodies have been produced that bind to DNA\textsuperscript{50, 55-56}. Although not as common, some MAbs have been produced that bind specifically to methylated sequences or even methylated nucleotides in DNA\textsuperscript{26, 57-64}. Even less common, MAbs have been produced that bind to methylated adenosine or deoxy methylated adenosine, in which the adenine has been methylated at the 1, 3, or 6 position\textsuperscript{26, 59, 64-65}. Only one of these MAbs detects deoxy methylated adenosine that has been methylated in the naturally occurring 6 position\textsuperscript{26}.

Hydrophobic interactions and van der Waals interactions are the two main types of interactions that occur between a paratope (the specific amino acids that interact with a binding site of an antigen) and its target binding site (an epitope)\textsuperscript{31}. The variable regions of antibodies are what allow for a diversity of paratopes. Immunoglobulin G antibodies belong to a class of antibodies produced by plasma B cells\textsuperscript{31}. Immunoglobulin G antibodies appear to be used the most for binding to DNA. Although IgG antibodies are able to bind to an oligonucleotide that is twenty nucleotides long (a 20-mer), IgG antibodies were found to more effectively bind to a 40-mer\textsuperscript{56, 66}.

Furthermore, anti-DNA IgG antibodies produced against DNA through immunization tend to be structure specific\textsuperscript{55-56, 67-69}. The affinity of an anti-double-stranded (ds)-DNA antibody is often much greater for dsDNA than single-stranded (ss) DNA and vice versa for an anti-ssDNA antibody, showing low cross-reactivity\textsuperscript{55-56}. The influence of affinity on avidity or vice versa tends to depend on the particular antibody\textsuperscript{43, 56, 66}. For instance, the avidity of an antibody is affected by the valency of the antibody, the affinity of each Fab, and the coordination between the Fabs of the antibody. Likewise, the affinity of an antibody measured as a whole unit may have either
synergistic or obstructive effects due to coordination between the Fabs or a lack thereof resulting from steric constraints, respectively. Thus, the affinity measured for the binding of an antigen to a Fab in a complete antibody may be stronger or weaker than the binding affinity of an antigen to a Fab alone. Each IgG is divalent, in which it has two variable binding domains that can each bind to a sequence in the DNA. In the case of a 20-mer, generally univalent or single variable domain binding occurs; however, in the case of a 40-mer divalent binding can occur.

**Brief Description of Typical Anti-DNA Interactions**

Generally, anti-DNA binding interactions fall into five main categories: \(\pi\)-stacking interactions, hydrogen bonds, charge-charge interactions, polar interactions, and hydrophobic, non-stacking interactions. \(\pi\)-Stacking interactions are the strongest non-covalent interactions producing typically \(\geq 9\) kcal/mol. However, a \(\pi\)-stacking interaction of a tyrosine residue with adenine in a catalytic site was reported as producing approximately -2 kcal/mol of energy. Hydrogen bonds are the second strongest type of intermolecular forces producing -2 to -10 kcal/mol of energy. Hydrogen bonds increase in strength linearly with aromatic stacking interaction. Other interactions also can increase H-bond strength.

Charge-charge interaction strengths are determined by Coulomb’s law: 

\[
E = \frac{kq_1q_2}{Dr}
\]

where \(k\) is a proportionality constant, \(q_1\) and \(q_2\) are charges of two participating atoms, \(D\) is the dielectric constant, and \(r\) is the radius between the two participating atoms. In aqueous solutions, charge-charge interactions generally contribute in a similar energy range as H-bonds. Polar contacts occur through dipole-dipole forces,
which are typically weaker than H-bonds or charge-charge interactions, producing -1 to -3 kcal/mol of energy. Hydrophobic contacts occur through London dispersion forces, which are the weakest of the five mentioned forces producing -0.01 to -2 kcal/mol of energy. Aromatic-aromatic hydrophobic contacts produce approximately -1 to -2 kcal/mol. In a study using AutoDock-4.0, H-bonds were found to produce only -0.6 kcal/mol per bond and dispersion/repulsion forces were found to produce only -0.3 to -0.5 kcal/mol per nonhydrogen contact.

**Structural Aspects of IgGs**

Hinges of IgGs tend to be flexible, allowing movement and rotation of the hinges. As a result, the hinges allow Fab fragments to attain more positions than merely open or shut like a door hinge. Hinge length and domain variability were found to limit the reach of the Fab fragments. Shorter IgG hinges tend to allow for a Y-shaped structure, while longer IgG hinges tend to allow for more of a T-shaped structure.

Only three complete structures of IgG mAbs have been reported: MAb 61.1.3, a murine IgG1, MAb 231, a murine IgG2a, and IgG1 b12, a human IgG1. Among antibodies from the four murine IgG subclasses (IgG1, IgG2a, IgG2b, and IgG3), IgG1 antibodies possess the shortest hinges and are considered the most rigid.

Interestingly, the 3.2 Å crystal structure of MAb 61.1.3 has one C\textsubscript{H}2 domain that is considerably more mobile than the other. In fact, the highly mobile C\textsubscript{H}2 domain of MAb 61.1.3 is more mobile than both C\textsubscript{H}2 domains of MAb 231. This finding suggests that murine IgG1 antibodies have extensive segmental flexibility simply in a different manner than other IgGs. The MAb 61.1.3, with its Fabs much closer together, is...
a more compact antibody than MAb 231. The angle between Fabs in MAb 61.1.3 is 115° making a distorted Y shape, while the angle between Fabs in MAb 231 is 172° and Fabs in IgG1 b12 is 180° making a distorted T shape and a T shape, respectively. The specific angles between Fabs may be related to the size of an antigen. For instance, the MAb 61.1.3 binds a relatively small antigen, Phenobarbital, compared to MAb 231, which binds to an antigen on the surface of canine lymphoma cells, and IgG1 b12, which binds to the CD4-binding site of human immunodeficiency virus-1 (HIV-1) gp 120.

**Structural Aspects of Anti-Nucleic Acid Fabs and Paratope Interactions**

Over 300 crystal structures of antibodies or antibody fragments are in the Protein Data Bank (PDB). Of all these structures, only four are Fab fragments of anti-nucleic acid MAbs. Two of these Fab fragments (BV04-01 and DNA-1) bind to deoxythymidine (dT) sequences in ssDNA, one of these Fab fragments (ED-10) binds to ssDNA sequences containing dT-deoxycytosine (dC), and the other Fab fragment (Jel 103) binds to RNA. Comparison of BV04-01 and DNA-1 provide a good example of the similarities and differences commonly found in nucleotide specific binding interactions involved in anti-ssDNA binding.

A 2.0 Å crystal structure of unliganded BV04-01 and a 2.66 Å crystal structure of BV04-01 complexed with a deoxythymidine trimer (dT₃) revealed that the active-site must expand to accommodate the antigen. Stacking interactions and hydrogen bonding between BV04-01 residues and thymine-2 (T2) were the critical binding interactions. Tyrosine 32 of the light chain (Tyr L32) and tryptophan 100a of the heavy chain (Trp H100a) of BV04-01 were the residues involved in the stacking interactions.
Nevertheless, hydrogen bonding and van der Waals contacts between polar residues in BV04-01 and the phosphate groups, especially phosphate-1, in dT₃ contributed to binding by stabilizing the phosphodeoxyribose backbone⁴². van der Waals contacts between the deoxyribose and Trp H100a, Arg H52, His L27d, and His L93 also contributed to stabilizing the phosphodeoxyribose backbone. Ion pairing occurred between Arg H52, which is found in the CDR-H2, and phosphate-2 of dT₃. Typically, CDR-H3s of anti-DNA MAbs are relatively rich in Arg residues; however, BV04-01 binds to ssDNA not dsDNA⁴².

DNA-1 and BV04-01 show similarities and differences in the types of interactions that bind to oligo(dT) polynucleotides. A 2.1 Å crystal structure of DNA-1 complexed with dT₅ revealed that tyrosine side-chains intercalating between thymine bases were responsible for stacking interactions between the Tyr side-chains and the thymine bases⁵⁰. The stacking interactions were proposed to be important in generating the experimentally observed negative enthalpy of binding⁵⁰, 84-85. The stacking interactions are believed to generate a negative enthalpy through the desolvation of the interface between binding pocket and nucleotide base⁵⁰. Stacking interactions due to Tyr L32, Tyr L49, Tyr H100, and Tyr H100A are the most critical for binding to dT₅ or dT₃⁴⁹-⁵⁰. As a result, the primary source of binding of dT₅ or dT₃ to DNA-1 is through stacking interactions with the thymine bases, while binding of dT₃ to BV04-01 involved a more even distribution of interactions between the thymine bases and the phosphodeoxyribose backbone.

Interestingly, the two Fab fragments of DNA-1 in crystal structures are asymmetrical, in which most of the stacking interactions are made by CDR-H3 of Fab1
and CDR-L1 and CDR-L2 of Fab1. The CDR-H3 is a peptide about two bases long and comprises one side of the narrow groove that interacts with three thymine bases. The CDR-L1 and 2 comprises the other side of the narrow groove. DNA-1 binds to a deoxythymidine 15-mer (dT$_{15}$) or longer oligo(dT) with high affinity. Nevertheless, DNA-1 also binds to smaller sequences of oligo(dT), such as dT$_5$ and dT$_3$, but with lower affinity than it binds to dT$_{15}$. 

**Antibody Variable Regions**

Antibody V regions show high similarity between sequences (at least 50% similarity between sequences) $^{90}$. The FRs of V domains have been shown to have rather conserved structures and five of the six CDRs have been found to follow canonical patterns $^{44-45, 91-92}$. Since CDRs often overlap in range with loops and show more variability than the FRs, they require separate consideration during modeling $^{45}$. Computer modeling of the V region of V-88, an IgG1κ anti-ssDNA antibody, has been done based on known crystallographic structures of other antibodies $^{93-94}$. Antibodies that share the highest degree of identity with sequences for the V-88 $\text{V}_\text{H}$ and $\text{V}_\text{L}$ were searched for on the PDB $^{93}$. V-88 showed a high sequence identity with the anti-fluorescein Ab 4-4-20 for both the $\text{V}_\text{H}$ (67%) and the $\text{V}_\text{L}$ (93%), serving as a template for the V-88 model. The final residues of FR-H3 and the entire FR-H4 were modeled from BV04-01, which had nearly identical sequences to V-88 in these regions. The three light chain CDRs and the CDR-H1 were all constructed according to canonical structures found in 4-4-20 $^{93}$. The CDR-H2 was different in length than all four of the observed canonical structures for this region.
As a result, the CDR-H2 of V-88 was partially designed from the canonical structures of the CDR-H2 of 4-4-20 and BV04-01. A conformational search was performed to determine the structure of the remaining sequence. No canonical forms have been observed for CDR-H3 of V regions. As a result, the structure for the CDR-H3 of V-88 was based on the structure of the CDR-H3 from BV04-01 since the final residues of FR-H3 and the entire FR-H4 for V-88 and BV04-01 share considerable sequence identity. In addition, the sequence for CDR-H3 of V-88 is only two residues longer than the sequence for CDR-H3 of BV04-01.

Previous Research from the O.J. Lopez Laboratory

Polyclonal and monoclonal antibodies have been used in detection of methylated sequences within DNA. Rabbit anti-6mA antisera detected deoxy-N6-methyladenosine (d6mA) in DNA, but did not detect sequences with deoxy-5-methylcytidine (d5mC) or deoxy-4-methylcytidine (d4mC) as demonstrated by an immunochemical genotyping Southern blot. This experiment demonstrated the ability of antibodies to distinguish among specific nucleotides. A monoclonal antibody that specifically binds to N6-methyladenosine (6mAos) has been produced jointly by Dr. Osvaldo Lopez and Dr. Michael Nelson by inoculation of a murine spleen with 6mAos conjugated to hemocyanin.

The conjugation of 6mAos was performed according to methods previously described. The immunization of BALB/c mice with 6mAos-hemocyanin conjugate and generation of a hybridoma that produced a MAb against 6mAos was previously described. The anti-6mAos MAb was purified and isotyped to be IgG1κ. An enzyme-
linked immunoassay (ELISA) was done to test the resulting hybridomas for production of antibodies that detect *Chlorella* virus NY-2A (CV NY-2A), which is a virus that contains a high proportion of methylated nucleotides$^{96}$; approximately 37% of adenines in CV NY-2A are methylated in the N$^6$ position$^{100}$. Figure 2 shows a diagram of the overall process of initial hybridoma production.
Figure 2. Diagram illustrating the main steps for initial production of hybridomas and, in turn, monoclonal antibodies. Abbreviations: HGPRT<sup>−</sup>, hypoxanthine-guanine-phosphoribosyl transferase negative; PEG, polyethylene glycol; and HAT, hypoxanthine-aminopterin-thymidine.
An immunogenotyping ELISA showed that the 7C7:C5 MAb could detect a single d\textsuperscript{6m}A in 100 ng of a 97-mer ssDNA strand\textsuperscript{26}. An ELISA utilizing the high amount of d\textsuperscript{6m}A (~34,000 d\textsuperscript{6m}A per strand of DNA) present in CV NY-2A genomic DNA revealed that the 7C7:C5 MAb from ascites bound with twenty-seven fold greater intensity to the CV NY-2A DNA than to the DNA sequences that did not contain d\textsuperscript{6m}A\textsuperscript{96}. Competition ELISA revealed that the 7C7:C5 MAb binds to deoxy-N\textsuperscript{6}-methyladenosine-triphosphate (d\textsuperscript{6m}ATP), which, in turn, competitively inhibits binding to sequences with d\textsuperscript{6m}A in DNA\textsuperscript{96}. The competition ELISA also demonstrated that the 7C7:C5 MAb does not competitively bind to deoxy-5-methylcytidine-triphosphate (d\textsuperscript{5m}CTP) or to deoxyadenosine-triphosphate (dATP), resulting in no inhibition of the 7C7:C5 MAb binding to d\textsuperscript{6m}A in DNA. The results of the immunochemical genotyping ELISA, the CV NY-2A ELISA, and the competition ELISA suggest that the 7C7:C5 MAb specifically recognizes d\textsuperscript{6m}A in DNA\textsuperscript{26,96}.

**Summary of Objectives Accomplished Herein**

The 7C7:C5 hybridoma cell line has been used to produce a monoclonal antibody that detects d\textsuperscript{6m}A in DNA\textsuperscript{26,96}. It is the only MAb that has been isolated for detecting d\textsuperscript{6m}A. Unfortunately, the anti-\textsuperscript{6m}A\textsubscript{os} 7C7:C5 MAb has yet to be fully characterized. The genes for the V regions were previously unknown and, thus, the genes were obtained by reverse-transcription polymerase chain reaction (RT-PCR). Subsequently, the PCR product for each of these genes was cloned into a vector and the resulting plasmid was submitted for DNA sequencing. Upon obtaining the complete DNA sequences for the V regions, the amino acid sequences of the V regions were determined for the purpose of
constructing a three-dimensional (3D) molecular model of the variable fragment (Fv) of the 7C7:C5 MAb. The 3D model of the 7C7:C5 Fv was then used to predict the interactions involved in the binding of the 7C7:C5 MAb to $d^{6m}A$ in DNA. Analyses of docking experiments with the molecular model were used to better understand the specificity of the 7C7:C5 MAb for $d^{6m}A$ and its future usability in DNA and potentially RNA genotyping. Lastly, its potential usage as a carrier of DNA and RNA is also discussed.
MATERIALS AND METHODS

Obtaining the 7C7:C5 MAb

Hybridoma cells of the 7C7:C5 cell line that produce the anti-$^{6\text{m}}$Aos MAb were grown on RPMI cell medium/20% fetal calf serum (FCS) for two to three weeks in a 37°C CO$_2$ incubator. The cells were pelleted by centrifuging at 1,000 x g for ten minutes. The supernatant containing the 7C7:C5 MAb was extracted and placed in several 15 mL sterile tubes. The supernatant was stored at -20°C for later use.

Transformation and Growth of Escherichia coli with pUC-18 or pUC-19 Plasmid

Luria-Bertani (LB) agar broth was made using 12.5 g of LB broth and 7.5 g of agar diluted to 500 mL with distilled water. The LB agar broth was autoclaved and trihydrate ampicillin (Sigma) was added to make a final composition of 50 µg/mL of ampicillin in LB agar broth when the broth reached 45-50°C. The solution was homogenized by mixing. The LB agar broth with ampicillin (LBA) was poured onto Petri plates, using aseptic technique. The LBA medium was allowed to solidify overnight (~16 hours). LB plates without ampicillin were made using the same methods, excluding the addition of ampicillin. The plates were inverted and stored at 0-4°C until needed.

Transformation was performed using One Shot TOP10 Chemically Competent E.coli (Invitrogen). Two vials of frozen TOP10 E.coli cells (Invitrogen) were obtained. After thawing the vials in ice, 5 µL of 10 pg/µL of pUC-19 in 5 mM Tris-HCl, 5 mM EDTA, pH 8, was added to one of the vials. Both vials were kept in ice for thirty
minutes. The vials were incubated at 43°C in a dry bath with mineral oil. The vials were then placed in ice for two minutes. To culture the *E.coli*, 250 µL of SOC medium (Invitrogen) was added to each vial. The vials were then incubated at 37°C for thirty minutes in a warm room. Following the incubation, 50 µL of culture from the vial containing TOP10 *E.coli* with pUC-19 was plated onto an LBA plate and a LB plate. Also, 50 µL from the vial containing TOP10 *E.coli* without pUC-19 was plated onto an LBA plate and a LB plate as a negative control to ensure that the *E.coli* or the medium were not faulty. The plates were incubated overnight (~18 hours) at 32°C. The plates were inverted and stored at 0-4°C until isolation of pUC-19 from transformed *E.coli*.

**Isolation of Plasmid for Blotting Methods**

(1) Lab bench miniprep\(^\text{101}\), (2) Fast Plasmid Mini (Eppendorf), and (3) PureYield Midiprep System (Promega) were used to obtain pUC-18 or pUC-19 plasmid. In cases in which the concentration of DNA or purity of DNA was too low, a phenol/chloroform/isoamyl alcohol extraction of proteins and precipitation of DNA with 0.3 M sodium acetate and two volumes of 95% ethanol at -80°C for 2.5 hours or at -20°C overnight was performed\(^\text{101}\). Approximate DNA concentrations were determined by ultraviolet (UV) spectrophotometry, based on a concentration of 50 µg/mL of DNA having an absorbance of 1.000 at 260 nm. Purity of DNA was also determined by calculating a ratio of absorbance at 260 nm/absorbance at 280 nm. A ratio of 1.8-2.0 was considered good purity, with 1.8 being the best purity\(^\text{101}\).
Methylation of Plasmid DNA with EcoRI Methylase

EcoRI methylase, 32 mM (400X) S-adenosylmethionine, and 10X NE EcoRI methylase buffer were purchased from New England Biolabs. Final composition of the methylation solution/mix included 1X NE EcoRI Methylase Buffer (50 mM NaCl, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0 at 25°C), at least 1 µg of unmethylated DNA, 800 µM SAM, and 40 U of EcoRI Methylase in storage solution (200 mM NaCl, 100 mM KPO₄, pH 7.4, 0.1 mM EDTA, 10 mM BME, 200 µg/mL BSA, and 50% glycerol). The final composition was attained by diluting DNA methylation solution with autoclaved distilled water to an appropriate final volume. The DNA methylation solution was incubated at 37°C in a water bath for one hour and fifteen minutes.

A protection assay was performed to determine the extent of methylation. Two DNA samples (one is presumed to be methylated and the other is unmethylated) of equal volume and concentration were treated with EcoRI restriction endonuclease (RE). The final composite for the EcoRI RE solution/mix was at least 1 µg of DNA (methylated or unmethylated), 1X Buffer Y⁺/Tango with BSA, and 5 U of EcoRI RE. The final composition was attained by diluting DNA methylation solution with autoclaved distilled water to an appropriate final volume. The EcoRI RE solutions/mixes were incubated at 37°C in a water bath for thirty-five minutes. Untreated (unmethylated and undigested) DNA was used as a negative control. To view band movement, 2 µL of bromophenol blue 6x loading dye was added to 10 µL of each DNA sample. Each sample was mixed and loaded onto different lanes on a 1% agarose gel (containing ≥0.1 µg/mL of ethidium bromide). Samples underwent gel electrophoresis at 72 V. Results of the protection assay were analyzed by the appearance of bands upon short wavelength UV exposure.
Dot Blot

The general procedure for dot blots was designed based on methods previously described. Adjustments to the protocol for dot blots were made in communication with Lopez, O.J.

A solution of 1X phosphate buffered saline (PBS), pH 7.5, was prepared from a 1:10 dilution of 10X PBS (1.5 M NaCl, 0.16 M of Na₂HPO₄, and 0.04 M of NaH₂PO₄; pH was adjusted using 10 M NaOH), pH 6.9-7.0. A solution of 10% nonfat milk in 1X PBS, pH 6.65, was prepared from 1 g of Sanalac nonfat dry milk diluted to 10 mL with 1X PBS, pH 7.5. Suitable sizes of polyvinylidene difluoride (PVDF) membranes were cut for each dot blot and transferred to Petri plates, in which they fit securely. Tweezers were used to move and transfer the membrane. Three edges of the membrane were cut for orientation. Circles were drawn in pencil to show where to distribute each dilution of DNA.

Separate rows or columns of circles were made for methylated and unmethylated DNA. Each DNA (methylated or unmethylated) dilution was prepared in TE (10 mM Tris, 1 mM EDTA), pH 8.2, in 1.5 mL polypropylene tubes. DNA dilutions were then heated in a water bath at 95°C for five minutes to denature the DNA. Immediately after heating DNA dilutions were put on ice for two minutes to prevent renaturation. DNA dilutions were centrifuged for five seconds in a tabletop centrifuge to collect all liquid to the bottom. An aliquot of 10 µL of each dilution of DNA was pipetted onto a PVDF membrane. The DNA dots were allowed to air dry at 21-37°C for a minimum of two hours. After DNA dots appeared visibly dried and immobilized on the membrane, DNA was fixed to the PVDF membrane by exposure to UV irradiation for two minutes. The
membrane was then inverted and immersed in milk blocking solution (10% nonfat milk in 1X PBS, pH 6.65). The membrane was incubated in milk blocking solution overnight (15-18 hours) at 0-4°C.

The milk blocking solution was discarded and the membrane was washed five times with 1X PBS, pH 7.5. The 7C7:C5 monoclonal antibody (MAb) in RPMI/10% FCS was thawed. The membrane was inverted and immersed in 1.5 mL of 7C7:C5 MAb solution and then incubated at room temperature for one hour and fifteen minutes with rotating movement. This period of incubation allows for sufficient binding of 7C7:C5 MAbs to DNA that has been fixed to the membrane. The 7C7:C5 MAb solution was discarded after incubation and the membrane was washed five times with 1X PBS, pH 7.5.

A vial containing 0.1 mg of lyophilized goat polyclonal anti-mouse IgG conjugated to alkaline phosphatase (amIgG-AP)/goat serum or bovine serum albumin (BSA) (KPL) was dissolved in 50% glycerol (0.5 mL of nuclease-free water and 0.5 mL of glycerol) before its use. Next, 3 µL of the amIgG-AP was mixed with 1.47 mL of milk block solution, pH 6.65, to make a 1/500 dilution of the amIgG-AP. The membrane was inverted and immersed in 1/500 amIgG-AP solution and then incubated at room temperature for one hour and fifteen minutes with rotating movement. This period of incubation allows for sufficient binding of the amIgG-APs to 7C7:C5 mAbs.

After incubation, the 1/500 amIgG-AP solution was discarded and the membrane was washed five times with 1X PBS, pH 7.5. The membrane was inverted and immersed in 1.5 mL of 0.21 g/L of 5-Bromo-4-chloro-3-indoyl-phosphate/0.42 g/L of nitroblue tetrazolium (BCIP/NBT) phosphatase substrate (1-Component) (KPL) in an organic
base/Tris buffer. The membrane was incubated in substrate for twenty-five minutes at room temperature with rotating movement. This period of incubation allows for sufficient reaction time for alkaline phosphatases to catalytically remove phosphate groups from the BCIP/NBT substrates causing the BCIP/NBT solution to change from yellow/greenish-yellow to purple in places where a reaction occurred. The substrate was discarded after incubation. The membrane was inverted and immersed in autoclaved distilled water for 20-30 seconds to halt any remaining reactions. The membrane was allowed to air dry overnight in the dark and stored in the dark to prevent stain from fading.

Slot Blot

SSC powder (43.825 g of NaCl and 22.05 g of sodium citrate) (Vysis) was diluted to 250 mL in distilled water to make 20X SSC, pH 7.6. A solution of 2X SSC was prepared by a 1:10 dilution of 20 X SSC, pH 7.6, in distilled water. Essentially the same protocol was performed for the slot blot as was done for the dot blot, with the following exceptions: (1) Schleicher & Schuell (S&S) nitrocellulose membrane was used in place of PVDF membrane, (2) membrane sizes had to be larger to accommodate the wider areas of slits versus dots, (3) little pencil markings rather than circles indicated the approximate location for the start of each slit, (4) 10 µL of each DNA dilution in TE was diluted to 500 µL in 2X SSC, (4) the S&S Minifold I Slot-Blot Filtration Manifold (in place of air drying) was used to immobilize the DNA samples onto the nitrocellulose membrane using the procedure described in the instruction manuals for the apparatus, (5) DNA was fixed to the nitrocellulose membrane while still damp by using the auto-cross-
linking (exposure to 1,200 J of UV at 254 nm for approximately thirty seconds) function on the 1800 Stratalinker UV Crosslinker (Stratagene) and (6) larger amounts of 7C7:C5 MAb solution, 1/500 amIgG solution, and BCIP/NBT phosphatase substrate (1-Component) were used due to larger membrane sizes.

Generally the slot blot membranes were at least twice as large, so 2-5 mL of each of the antibody solutions and 2-5 mL of substrate worked well. In the case of the optimized slot blot, the membrane was inverted onto a Kodak DS UV Camera and a picture of the membrane was taken by shining UV light through the membrane. Band intensities were determined for all bands that appeared visible using Kodak DS UV Software. Band intensities were determined for equal areas from the left center to the middle center of each band, so as to avoid measuring any pencil markings and to make band intensity (not band intensity and area) the variable that was measured. The general scheme behind the slot blot is shown in Figure 3.

![Slot blot schematic](image-url)

Figure 3. Slot blot schematic.
**Total RNA Isolation**

For total RNA isolation, a customized version of the TRIzol method was used. TRIzol Reagent (Invitrogen), a monophasic solution containing phenol and guanidine isothiocyanate, is an improved version of the solution used by Chomczynski and Sacchi for the “Single-Step Method of RNA Isolation”\(^\text{102}\). Chomczynski and Sacchi used 4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol mixed sequentially with 0.1 mL of 2 M sodium acetate, pH 4, 1 mL of phenol saturated with water, and 0.2 mL of chloroform-isoamyl alcohol mixture (49:1) for the isolation of total RNA. A modified form of the single-step method for the isolation of total RNA was used according to the instruction manual for TRIzol (Invitrogen) along with some additional modifications not included in the manual for TRIzol.

7C7:C5 Hybridoma cells were originally grown in RPMI 1640/10% FCS and cells/mL of medium were counted under a microscope. These hybridoma cells were stored in RPMI 1640/10% FCS/8% dimethylsulfoxide (DMSO) in a cryotube at greater than -70°C. Each cryotube contained at least 2 x 10\(^6\) cells but no more than 5 x 10\(^6\) cells. The cells were thawed on ice and then immediately the 1 mL of cells in the cryotube was added by sterile pipet to 9 mL of 1X PBS, pH 7, in a sterile 15 mL plastic centrifuge tube. The cells were pelleted by centrifugation at 1,500 x g for five minutes and supernatant was carefully removed and discarded. Cell pellets were resuspended in 1mL of 1X PBS, pH 7, by gentle vortexing, so as to not break the cells. The cells in PBS were transferred
via pipet to a sterile 1.5 mL Eppendorf tube and the cells were pelleted by centrifugation at 8,000 x g.

The cells were lysed by adding 1 mL of TRIzol Reagent. The cell pellet was rigorously vortexed for two to three minutes to completely breakdown the pellet and homogenize the cell contents throughout the TRIzol. After vortexing, the cell contents were incubated in TRIzol for five minutes at room temperature to allow for nucleoprotein complexes to completely dissociate. The RNA was protected from ribonuclease activity by the presence of 4M/concentrated guanidine during this incubation\(^\text{102}\). To separate the organic phase and the aqueous phase, 0.2 volumes of chloroform was added to the TRIzol/RNA supernatant and mixed vigorously. Three minutes of incubation at room temperature allowed for separation of phases. To completely separate the phases, the RNA isolation mix was centrifuged (11,000 x g) for 20 minutes at 4°C. Cell contents were dispersed into three separate layers in TRIzol. The top layer is the aqueous phase and contains all the RNA; the middle layer is the interphase and contains both DNA and proteins; and the bottom layer is the red, phenol-choroform phase and contains both DNA and proteins.

After transferring the aqueous phase to a new 1.5 mL tube, 100 \(\mu\)L of 3M sodium acetate, pH 5.2, 30 \(\mu\)L of RNA-grade glycogen, and 870 \(\mu\)L of isopropyl alcohol was added to the aqueous phase to precipitate the RNA. The use of glycogen in the precipitation of RNA was found to be unnecessary, but the use of 3M sodium acetate resulted in greater yields of RNA. Following a ten minute period of incubation at room temperature, the RNA precipitation mix was placed at -20°C for a minimum of ninety minutes. The precipitated RNA was then centrifuged (11,000 x g) at 4°C for a total of \(\text{27}\)
thirty-five minutes. The RNA generally formed a barely noticeable gel-like pellet along the bottom of the microfuge tube. The supernatant was carefully removed by decantation so as not to disturb the pellet. The pellet was washed (without resuspension) in 1 mL of 70% ethanol. The RNA wash was centrifuged at 4°C for fifteen minutes at 6,000 x g. The supernatant was carefully removed by decantation. Centrifugation for a few seconds was used to collect any remaining supernatant followed by supernatant removal by pipet. The pellet was air dried for seven minutes. The pellet was then resuspended in 15-25 µL of nuclease-free water.

An aliquot of 5 µL of RNA in nuclease-free water (with 1 µL of 6X bromophenol blue loading dye added to it) was electrophoresed at 72 V for forty minutes on a freshly made 1% agarose gel, 0.1 µg/mL ethidium bromide in autoclaved 1X TAE. Gels for RNA gel electrophoresis were all prepared using clean, autoclaved glassware and equipment. The gel electrophoresis apparatus was cleaned with 10% bleach and autoclaved DI water to help prevent/limit nuclease contamination. The results of gel electrophoresis were analyzed under UV light.

Bands of high molecular weight (7-15 kb) RNA may appear. These bands are for mRNA and heterogeneous nuclear RNA (hnRNA). Two predominant bands (an 18S band, approximately 2 kb, and a 28S band, approximately 5 kb) of ribosomal RNA (rRNA) should appear. The 28S band should be brighter. The 18S band may also be comprised of a relatively small portion of mRNA due to the association of some mRNA activity with the 18S region\textsuperscript{103}. Also, a 12S region of RNA from rabbit globin was found to present some mRNA activity. Nevertheless, the major portion of biologically active/poly(A)-containing mRNA occurs as 9S RNA; however, a 9S band does not
generally appear on the gel due to the low amounts of poly(A)-containing mRNA in total RNA $^{103-104}$. The only RNA bands that appeared in all of the RNA isolation experiments using TRIzol method were the 28S and 18S bands and a high molecular weight band attributed to DNA contamination from the cells.

**Reverse Transcription Polymerase Chain Reaction (RT-PCR) of the 7C7:C5 V$_\kappa$ and V$_H$ Genes**

Reverse transcription polymerase chain reaction (RT-PCR) was used to produce complementary, copy DNA (cDNA) and then amplify cDNA. Reverse transcription polymerase chain reaction is comprised of two main processes: (1) cDNA synthesis and (2) cDNA amplification. The gene specific 3’-forward (reverse and complement to 5’→3’ template) primers listed in Table 2 were used to produce cDNA of the variable part of the light chain gene, excluding most of the C$_\kappa$, and cDNA of the variable part of the Fd of the heavy chain gene, excluding most of the C$_H$1, by reverse transcription.

The light chain cDNA produced includes the genetic sequence of part to all of the leader sequence of the kappa light chain (L$_\kappa$), the variable sequence for the variable region of the kappa light chain (V$_\kappa$), the joining sequence for the variable region of the kappa light chain (J$_\kappa$), and nucleotides 57-22 of the constant sequence of the kappa light chain (C$_\kappa$). The heavy chain cDNA produced includes the genetic sequence of part to all of the leader sequence of the gamma 1 heavy chain (L$_\gamma$), the variable sequence for the variable region of the gamma 1 heavy chain (V$_\gamma$), the diversity sequence for the variable region of the gamma 1 heavy chain (V$_H$), the diversity sequence for the variable region of the gamma 1 heavy chain (D), the joining sequence for the variable region of
the gamma 1 heavy chain (J_{\text{H}}), and nucleotides 51-16 of the constant region 1 of the gamma 1 heavy chain (C_{\text{H}1}).

The amount of cDNA was amplified using polymerase chain reaction (PCR) \(^{105}\). In addition to increasing the amount of cDNA, the amplification process converts the single-stranded cDNA produced by RT into double-stranded cDNA (a more stable form of cDNA). The amplification of cDNA requires the use of 5’-reverse (identical to 5’→3’ template) primers and 3’-forward primers. The setup used for cDNA synthesis is shown in Figure 4 and the setup used for cDNA amplification is shown in Figure 5.
(a) cDNA Synthesis of the $V_\kappa$

mRNA of the IgG1 Kappa Chain

\[
\begin{array}{|c|c|c|c|}
\hline
5' & \text{Leader} & V_\kappa & J_\kappa & C_\kappa & 3' \\
\hline
\end{array}
\]

Reverse Transcription (RT)

mRNA of the IgG1 of the Kappa Chain

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Leader} & V_\kappa & J_\kappa & C_\kappa \\
\hline
\end{array}
\]

cDNA of the IgG1 $V-C_\kappa$

(b) cDNA Synthesis of the $V_\text{H}$

mRNA of the IgG1 Fd Region of the Heavy Chain

\[
\begin{array}{|c|c|c|c|c|}
\hline
5' & \text{Leader} & V_\text{H} & D & J_\text{H} & C_\text{H1} & 3' \\
\hline
\end{array}
\]

Reverse Transcription (RT)

mRNA of the IgG1 Fd Region of the Heavy Chain

\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{Leader} & V_\text{H} & D & J_\text{H} & C_\text{H1} \\
\hline
\end{array}
\]

cDNA of the IgG1 $V-C_\text{H1}$

Figure 4. General design for cDNA synthesis of genes that code for the (a) $V_\kappa$ and (b) $V_\text{H}$ (Lopez, O.J. [communication], 2005).
Figure 5. General design for amplification of the (a) $V_\kappa$ and (b) $V_\lambda$ genes (Lopez, O.J. [communication], 2005).
The primers that were used for the synthesis of cDNA are listed in Table 2 and the primers that were used for amplification of cDNA are listed in Table 3. The 3’-primers listed in Table 2 were designed to be reverse and complement for hybridization at nucleotides 57-22 of the murine C\(\kappa\) chain and at nucleotides 51-16 of the murine C\(\text{H}1\). All MLALT and MHALT 5’-primers listed in Table 3 were designed for the murine kappa and gamma 1 chain leader sequences, respectively. MLALT1-3 contain nucleotides for ribosome binding sites (CACC and ACC) in bold. The 3’-primers (CkFORa and Cg1FORa) were designed to be reverse and complement to nucleotides 30-10 of the C\(\kappa\) and nucleotides 24-4 of the C\(\text{H}1\), respectively. The VH 5’-primers were designed for the amino terminus of the V\(\text{H}\). All APg1 5’-primers were designed for the murine gamma 1 chain leader sequences. AVg1 5’-primer was designed for the amino terminus of the V\(\text{H}\). ACg1AF 3’-primer was designed to be reverse and complement to nucleotides 18-1 of the C\(\text{H}1\) and nucleotides 1-6 of the carboxyl terminus of the V\(\text{H}\). ACg1AF 3’-primer was never used because Cg1FORa worked fine for amplification of the 7C7:C5 V\(\text{H}\) gene.

Table 2. Primers for synthesis of cDNA

<table>
<thead>
<tr>
<th>Light Chain</th>
<th>Heavy Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3’-primers</td>
</tr>
<tr>
<td>C(\kappa)</td>
<td>5’- TGGTGGAAGATGGATAC -3’</td>
</tr>
<tr>
<td>MC(\kappa)</td>
<td>5’- CAGTGGATAGACAGATGG -3’</td>
</tr>
</tbody>
</table>

* The listed 3’-primers are partially derived from 3’-primers previously used for cDNA synthesis of variable regions\textsuperscript{106}. All primers were synthesized by IDT DNA.
**Table 3. Primers for amplification of Vκ and V\(\text{H}\) genes**

<table>
<thead>
<tr>
<th>Light Chain</th>
<th>Heavy Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5' -primers</strong></td>
<td><strong>5' -primers</strong></td>
</tr>
<tr>
<td>MLALT1.RV</td>
<td>MHALT1.RV</td>
</tr>
<tr>
<td>MLALT2.RV</td>
<td>MHALT2.RV</td>
</tr>
<tr>
<td>MLALT3.RV</td>
<td>MHALT3.RV</td>
</tr>
<tr>
<td>MLALT4.RV</td>
<td>MHALT4.RV</td>
</tr>
<tr>
<td>MLALT5.RV</td>
<td>VH1.RV</td>
</tr>
<tr>
<td>MLALT6.RV</td>
<td>VH2.RV</td>
</tr>
<tr>
<td>5'-ACCATGGAGACAGACACACTCTCTGCTAT-3'</td>
<td>5'-ATGGRATGSGAGCTGTGGMATSCTCTT-3'</td>
</tr>
<tr>
<td>5'-ACCATGGATTTTTCAAGTGCCAGATTTTAC-3'</td>
<td>5'-ATGRACCTTGAGGTGCTGTTT-3'</td>
</tr>
<tr>
<td>5'-ATGRAGTCACAKACYCAGGTCTTYRTA-3'</td>
<td>5'-ATGGCTGTCCTGCTGCTGCTCTT-3'</td>
</tr>
<tr>
<td>5'-CACCAGKCCWRCAGYTCAGYTCGT-3'</td>
<td>5'-ATGACGRCTACWTYTYY-3'</td>
</tr>
<tr>
<td>5'-ATGAAGTGGCTGCTGCTCAGT-3'</td>
<td>5'-GCTAGTGTGACGTGGAGSAGTC-3'</td>
</tr>
<tr>
<td>5'-ATGATGAGTCCTGCCCAGTTC-3'</td>
<td>5'-TGAGTGTGCAGCGAGGAGSAGAC-3'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>3' -primers</strong></th>
<th><strong>3' -primers</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>CkFORa</td>
<td>Cg1FORa</td>
</tr>
<tr>
<td>5'- GATGGATACAGTGGTGACGC-3'</td>
<td>5'- GACAGATGGGGGTGTCGTTT -3'</td>
</tr>
</tbody>
</table>

* All MLALT and MHALT 5'-primers were partially derived from 5'-primers previously used for cDNA amplification of IgG variable regions \(^{106-107}\). MLALT4 contains a ribosome binding site (CACC) and MLALT1-2 contain partial ribosome binding sites (ACC) in bold. All VH 5'-primers were supplied by Lopez, O.J. The CkFORa and Cg1FORa 3'-primers were derived from 3'-primers previously used for cDNA amplification of IgG variable regions \(^{108}\). All A series primers (APg1, AVg1, and ACg1 primers) were designed by Nimani, A., based on known murine gamma 1chain leader sequences. The names of the primers that successfully amplified the 7C7:CS V\(\kappa\) and V\(\text{H}\) genes are underlined. All primers were synthesized by IDT DNA. IUB symbol and corresponding degeneracies are R = (A, G), Y = (C, T), M = (A, C), K = (G, T), S = (C, G), and W = (A, T).
Only reverse primers with the appropriate complementary sequences annealed with the 5’ leader sequences (sequences that code for the signal peptide in the immature antibody) of mRNA that codes for the 7C7:C5 MAb. Primers for the leader sequences were chosen in place of primers for the start of the V\(_\kappa\) or V\(_\Pi\) sequences because amplifying from the leader sequence prevents the loss or alteration of sequences that code for the amino terminus of the variable region. In the case of amplification of the V\(_\Pi\), reverse 5’-primers for the amino terminus (VH series) were also used in PCR reactions to attempt to amplify the 7C7:C5 V\(_\Pi\) gene because none of the MHALT series of reverse 5’-primers for the leader sequence resulted in successful amplification of the 7C7:C5 V\(_\Pi\) gene after repeated attempts.

Unfortunately, none of the VH series of primers successfully amplified the 7C7:C5 V\(_\Pi\) gene after repeated attempts. As a result, I designed the A (APg1, AVg1, and ACg1) series of primers for amplification of murine gamma 1 variable regions. The APg1 primers were designed based on existing leader sequences found on Genbank. These leader sequences were collected into groups based on sequence similarities allowing for one primer to be developed for a small group of similar leader sequences. Degeneracies were placed in areas of each primer that seemed appropriate, so as to increase the efficiency of annealing the primer with leader sequences of high diversity. The 3’-primer for amplification of the V\(_\kappa\) hybridizes to nucleotides 30-10 and the 3’-primer for amplification of the V\(_\Pi\) hybridizes to nucleotides 24-4.

Copy DNA synthesis of the V\(_\kappa\) and V\(_\Pi\) genes was performed using Moloney murine leukemia virus (M-MuLV) reverse transcriptase (RT) (Fermentas), according to the protocol in the product description for M-MuLV RT. Moloney murine leukemia
virus RT has the ability to use either RNA or DNA primers and RNA or single-stranded DNA templates for cDNA synthesis, although greater cDNA yield occurs with an RNA template\textsuperscript{109-110}. It has inherent ribonuclease H activity which selectively degrades RNA in RNA-DNA hybrids, but does not degrade ssRNA, ssDNA, dsRNA, or dsDNA\textsuperscript{109,111-112}.

A nuclease-free 1.5 mL Eppendorf tube was kept on ice and the following solutions—10 µL of total RNA in nuclease-free water from TRIzol extraction and 0.65 µL of each 10 µM 3’-primer for the kappa chain or the gamma 1 chain from Table 2—were added sequentially to the tube. The tube was heated at 70°C for six minutes and then chilled on ice for two minutes to anneal the primers. The following solutions were added sequentially to the tube: 4 µL of 5X Reaction Buffer, 2 µL of 10 mM deoxynucleotide triphosphate (dNTP) mix, and 0.7 µL of 40 U/µL Ribolock Ribonuclease Inhibitor (Fermentas) to inhibit ribonuclease A, B, and C activity. The tube was incubated at 37°C for five minutes. Then, 2 µL of 20 U/µL M-MuLV RT (Fermentas) was added to the tube to make a final solution containing 20 µL total.

The final contents of the cDNA synthesis solution were total RNA (unknown concentration), one set (κ or H chain) of 325 nM 3’-primers listed in Table 2, 1X Reaction Buffer, 1 mM dNTP, 1 U/µL Ribolock Ribonuclease Inhibitor, and 2 U/µL M-MuLV RT. The cDNA synthesis reaction mix was incubated at 70°C for ten minutes to allow for cDNA synthesis to occur. After incubation, the reaction mix was chilled on ice for two minutes to halt the reaction and conclude cDNA synthesis.

TrueStart \textit{Taq} Polymerase (Fermentas) is a recombinant \textit{Taq} DNA polymerase (\textit{Taq}Pol) with heat-labile blocking groups that have been chemically joined to amino
acids in the polymerase. These thermolabile inhibitors-switches allow for a “hot start” PCR, which prevents nonspecific amplification of fragments such as “primer dimer” artifacts by keeping the polymerase inactive until the initial denaturation at 95°C. TrueStart TaqPol has been shown to produce significantly higher PCR yields and specificity over Taq DNA polymerase (Fermentas).

Methods used for the polymerase chain reaction essentially followed the protocol outlined in the product description for TrueStart TaqPol from Fermentas. Volumes and amounts listed below are for single PCR reactions and are typically scaled according to the number of PCR reactions performed, in which as many PCR reagents and materials as possible were fit in one homogenized mix before dispersing the mix evenly into individual PCR tubes. The following ingredients were added to each nuclease-free PCR tube in sequential order: 10X TrueStart Taq Buffer, 10 mM dNTP mix, 25 mM MgCl₂, 10 µM of gene-specific 5’-reverse primer for the Lκ or LΗ listed in Table 3, 10 µM gene-specific 3’-forward primer for the 5’ end of the Cκ or CΗ1, and 2.5-5 µL of cDNA template, and 2.5 U of TrueStart TaqPol. The contents were diluted in the PCR tube to 50 µL with DEPC-treated water to make a final solution containing 1X TrueStart Taq Buffer, 0.2 mM dNTP mix, 2.5 mM MgCl₂, 100 nM mix of gene-specific 5’-reverse primers, 100 nM gene-specific 3’-forward primer, and 0.05 U/µL of TrueStart TaqPol. The contents were mixed and centrifuged for a few seconds. The contents of each PCR tube were then overlayed with 100 µL of mineral oil to help heat contents more evenly and prevent evaporation.

The cDNA amplification process was set for thirty-five cycles in a DNA Thermocycler 480 (Perkin Elmer Cetus). Following a single cycle initial denaturation at
95°C for ninety seconds, the cDNA was incubated at the following temperatures and corresponding durations per cycle for thirty-five cycles: 94°C for one minute to denature DNA, 55°C for one minute to anneal primers, and 70°C for thirty seconds to extend primer sequence into a cDNA sequence. After the thirty-five cycles of amplification, the cDNA was incubated at 72°C for fifteen minutes to allow for final extension. A 10 µL aliquot of the cDNA product (with 2 µL of 6X orange G loading dye added to the aliquot and mixed) was loaded on a 1% agarose, ≥0.1 µg/mL ethidium bromide gel.

O’RangeRuler™ 100 + 500 bp DNA Ladder (Fermentas) was loaded onto the gel to measure an approximate size of the PCR product. Gels were generally electrophoresed at 59 V for seventy minutes. The results were analyzed by exposure of the gel to UV.

The main PCR product obtained using the primers CkFORa and MLALT2.RV was double-stranded cDNA for the Lκ-Vκ-Jκ-Cκ” gene displayed in Figure 5(a). The PCR product for the Lκ-Vκ-Jκ-Cκ” should be 400-450 bp. The main PCR product obtained using the primers Cg1FORa and APg1AZ.RV was double-stranded cDNA for the LΗ-VΗ-D-JΗ-CΗ” gene displayed in Figure 5(b). The PCR product for the LΗ-VΗ-D-JΗ-CΗ” should be 425-475 bp. Analysis of cDNA products were performed under UV exposure. The entire RT-PCR procedure was performed separately for the Lκ-Vκ-Jκ-Cκ” and LΗ-VΗ-D-JΗ-CΗ” genes using primers corresponding to the appropriate chain listed in Tables 2 and 3.

Unfortunately, a naturally occurring aberrant kappa chain, or non-expressing kappa chain, is transcribed along with the functional kappa chain in hybridomas. Of particular interest is the aberrant kappa chain derived from SP2/0 MOPC21 myeloma cells. This strain of myeloma cells was used in the fusion process for generating the
7C7:C5 hybridoma and, thus, are responsible for the production of the aberrant kappa chain by the 7C7:C5 hybridoma. In order to eliminate the possibility of the 7C7:C5 V\kappa PCR product being part of the aberrant kappa chain gene, the 7C7:C5 V\kappa PCR product was digested with KpnI RE. Many V\kappa sequences contain KpnI sites, however, the SP2/0 MOPC21 aberrant kappa chain does not have a KpnI site in its variable gene. Digestions with KpnI RE were performed as follows: 8 \mu L of PCR product was mixed with 10 U of KpnI RE and a final 1X Tango/Y⁺ buffer in a 1.5 mL tube. The tube was then incubated in a 37°C water bath for seventy minutes. Results were analyzed by standard gel electrophoresis.

**Cloning of the 7C7:C5 V\kappa and V\H Genes into Plasmids**

The cloning process can be broken into three parts: the cloning reaction, the transformation, and the isolation and analysis of the plasmid. The PCR products for the 7C7:C5 V\kappa and V\H were cloned into separate pJet1/blunt Cloning Vectors using the GeneJET PCR Cloning Kit (Fermentas). The pJet1/blunt vector contains a toxic gene to \textit{E.coli}. However, upon receiving an insert, this toxic gene is split up and \textit{E.coli} can survive with that plasmid. As a result, the pJet1/blunt vector is a positive selection vector (a vector that only replicates in \textit{E.coli} if it contains an insert).

For each cloning reaction the following solutions were added to a nuclease-free PCR tube: 2X Reaction Buffer, 2 \mu L of PCR product (non-purified), 5 \mu L of nuclease-free water, and 1 \mu L of DNA Blunting Enzyme. The contents of this PCR tube were mixed by vortexing briefly and centrifuging for a few seconds. The mixture was incubated at 70°C for five minutes to allow for a blunting reaction of the PCR product
and then chilled on ice for one minute to halt the blunting reaction. The following solutions were added to the blunting mix: 50 ng of pJET1/blunt Cloning Vector and 5 U of T4 DNA Ligase.

The final ligation solution contained 1X Reaction Buffer, 1:10 dilution of PCR product, DNA Blunting Enzyme, 2.5 ng/µL pJET1/blunt Cloning Vector, and 0.25 U/µL of T4 DNA Ligase. The contents of this PCR tube were vortexed briefly and centrifuged for a few seconds to mix. The ligation solution was incubated at room temperature (22°C) for eight minutes. The PCR tube containing the ligation reaction was placed on ice for two minutes to halt the reaction. The pJET1 Vector inserted the PCR product between nucleotides 498-497 of the vector. The known sequence of the pJET1 Vector now included the sequence of the unknown L\(\kappa\)-V\(\kappa\)-J\(\kappa\)-C\(\kappa\)” or L\(\eta\)-V\(\eta\)-D-J\(\eta\)-C\(\eta\)” gene of the 7C7:C5 MAb, depending on which PCR product insert was used in that particular cloning reaction.

**Transformation and Growth of E.coli with Plasmids Containing the 7C7:C5 V\(\kappa\) and V\(\eta\) Genes**

The vectors with the L\(\kappa\)-V\(\kappa\)-J\(\kappa\)-C\(\kappa\)” or L\(\eta\)-V\(\eta\)-D-J\(\eta\)-C\(\eta\)” inserts were then used to transform competent E.coli cells to allow for selection, amplification, and isolation of the plasmid later on. Competent E.coli cells were prepared using TransformAid Bacterial Transformation Kit (Fermentas). Untransformed JM109 E.coli cells were grown on a sterile LB plate at 37°C for sixteen hours the night before transformation to have fresh colonies ready for the transformation process. A single colony of JM109 E.coli was selected and grown in 1.5 mL of C-Medium in a 25 mL sterile glass test tube at 205 rpm in a 37°C warm room for 3-3.5 hours. The transformation solution was prepared by
mixing 250 μL of T-Solution A with 250 μL of T-Solution B. The resulting T-Solution mix was kept on ice until use.

The culture of JM109 *E.coli* was transferred to a sterile 1.5 mL Eppendorf tube. The culture of JM109 *E.coli* was centrifuged at 13,200 x g for one minute at room temperature. Supernatant was discarded and the cell pellet was resuspended in 300 μL of T-Solution mix immediately followed by incubation on ice for five minutes. The cell suspension was centrifuged at 13,200 x g for one minute at room temperature. Supernatant was discarded and the cell pellet was resuspended in 200 μL of T-Solution mix. The resuspension was immediately followed by incubation on ice for five minutes.

For the transformation, a 10 μL aliquot of vector product was added to 100 μL of cell suspension in T-Solution mix in a 1.5 mL Eppendorf tube. The contents (110 μL of transformation mix) in the tube were gently mixed and the tube was incubated on ice for five minutes. The newly transformed JM109 were then spread across a prewarmed LB plate containing 50 μg/mL of ampicillin. The plate was incubated overnight for sixteen hours at 37°C. In addition to the 7C7:C5 transformation mix, generally, 55 μL of transformation mix was prepared with the pJet1/blunt vector containing (+)-control PCR product and 55 μL of transformation mix was prepared with (-)-control vector (pJet1/blunt vector with no insert) to ensure that the transformation was working properly and that only vectors with inserts survived. All transformation mixes were grown on separate LB-ampicillin (LBA) plates.

Only *E.coli* colonies that successfully incorporated the plasmid contained the ampicillin resistance gene and, thus, only they grew on the LBA medium. Next, a transformed colony was swiped and placed in 13 mL of LB broth containing 50 μg/mL of
ampicillin in a 25 mL sterile glass test tube. An untransformed colony was grown in a separate glass test tube containing LBA broth as a negative control to ensure that only transformed colonies were growing in the prepared LBA broth. The inoculated broth was incubated at 205 rpm at 37°C for 16 hours. The next day ~1.5 mL of successfully transformed culture was placed in eight sterile 1.5 mL Eppendorf tubes for mini-prep using a kit.

**Isolation and Analysis of Plasmids Containing the 7C7:C5 Vκ and VH Genes**

Plasmid DNA was isolated using the GeneJET Plasmid Miniprep Kit (Fermentas). The protocol for the GeneJET plasmid isolation was performed with at least a few changes and customizations to the protocol to get better plasmid isolation results. Each tube containing transformed culture was centrifuged at 8,000 x g for two minutes. The supernatant was discarded by decantation. The pellet was washed with 1X PBS, pH 7, vortexing gently so as to not resuspend the pellet but rather to lift the pellet from the bottom of the tube. The pellet was centrifuged for one minute at 12,000 x g. One more wash with PBS followed by centrifugation was performed, only if the pellet still appeared dirty.

GeneJET plasmid isolation was adjusted from the standard protocol for the next steps using the following customizations: the pellet was vortexed for resuspension in the Resuspension Solution, the resuspended pellet was then lysed three to four minutes after mixing with the Lysis Solution, a few minutes of room temperature incubation were allowed for the white precipitate to form after mixing with the Neutralization Solution, and the white precipitate that formed was centrifuged for ten minutes at 12,000 x g to
help keep the precipitate in tact against the walls of the 1.5 mL Eppendorf tube while
decanting the DNA supernatant into the GeneJET Spin Column. The remainder of the
GeneJET Miniprep protocol from Fermentas was followed.

A 5 µL aliquot of the plasmid obtained was loaded on a 1% agarose gel, ≥0.1
µg/mL ethidium bromide along with single and double digested samples of the plasmid.
The double digestion of the plasmid was performed as follows: A 14 µL aliquot of
plasmid was digested with two FastDigest Units (FDU) of FastDigest (FD) XhoI
(Fermentas) and FD XbaI (Fermentas) restriction endonucleases (RE)s at a concentration
of 1 FDU/µL per RE in a 1X FD buffer. The digestion was performed by incubation of
the digestion mix in a nuclease-free 1.5 mL Eppendorf tube in a 37°C water bath for one
hour.

As additional controls, single digested aliquots of plasmid (one digested with FD
XhoI and the other digested with FD XbaI) were loaded onto the gels to ensure that the
enzymes were working properly in the digestion reactions and that the insert was not
being digested by either of the REs. Each sample contained a final concentration of 1X
orange G loading dye before loading samples onto a gel. An aliquot of O'RangeRuler™
100 + 500 bp DNA Ladder (Fermentas) was loaded onto the gel for band measurements.
The gel was electrophoresed at 59 V for one hour. The pJET1 vector contains an XbaI
site at nucleotide 503 and an XhoI site at nucleotide 478 on opposite sides of the plasmid
insertion site and, thus, the inserted gene is excised along with twenty-five additional
nucleotides when treated with FD XbaI and XhoI REs.

For plasmids cloned with Vκ inserts, a KpnI digestion was performed to check for
linearization of the plasmid using the following mix: 8 µL of plasmid DNA and 10 U of
KpnI RE in a final 1X Tango/Y+ buffer. The digestion was performed by incubation of the reaction mix in a 37°C water bath for seventy minutes. The pJET1 vector does not contain a KpnI site, so digestion depends on the presence of a KpnI site in the insert. Results were analyzed by standard gel electrophoresis as previously described in the Materials and Methods for PCR products.

**Preparation of Plasmids Containing the 7C7:C5 Vκ and VΗ Gene Inserts for Sequencing**

The plasmids were prepared for DNA sequencing at the University of Michigan DNA Sequencing Facility. The concentration of plasmids were measured as 1:10 dilutions in 10 mM Tris-Cl, pH 8, in a 100 μL quartz cuvette for Shimadzu spectrophotometers. An aliquot of 100 μL of 10 mM Tris-Cl, pH 8, was used as a reference and a blank for measurements. All plasmid samples were spectrophotometrically measured at absorbance 260 nm and 280 nm on a UV3101PC UV-VIS-NIR Scanning Spectrophotometer using UV Probe v.2.10. A purity (A260/A280 ratio) of 1.7-1.9, of which 1.8 is the best, was attained for all plasmids sent for sequencing.

Additional purification and concentration of plasmids was typically achieved by phenol/chloroform/isoamyl alcohol extraction of impurities followed by precipitation with 2 volumes of 95% EtOH and 0.1 volumes of 3M Na(OAc), pH5.2. The plasmids were diluted to 70-80 ng/μL when possible, although plasmids of approximately 50 ng/μL were also sent to the University of Michigan DNA Sequencing Facility when higher concentrations were not available. Generally, these less concentrated plasmids did not result in as high quality sequences as plasmids in the 70-80 ng/μL range of
concentration. The pJet1 forward and reverse primers were diluted in separate sterile 1.5 mL Eppendorf tubes.

Analysis of the Obtained DNA Sequences for the 7C7:C5 V_κ and V_Η Genes

Upon receiving the DNA sequences of the plasmids with the L_κ-V_κ-J_κ-C_κ” and L_Η-V_Η-D-J_Η-C_Η” genes, the DNA sequences for the L_κ-V_κ-J_κ-C_κ” and L_Η-V_Η-D-J_Η-C_Η” genes were first translated using the translate tool in the Expert Protein Analysis System (ExPASy)\textsuperscript{120}. The DNA sequences and corresponding amino acid translations were then processed in a National Center for Biotechnology Information (NCBI) Blat and pBlast to search for areas of identity with other murine IgG1 variable genes and regions, respectively.

The beginning and ending of the complete DNA sequence for the L_κ-V_κ-J_κ-C_κ” and L_Η-V_Η-D-J_Η-C_Η” genes were located based on two accounts: 1) by finding at least part of the DNA sequences for the L_κ and L_Η and for the C_κ” and C_Η1” and 2) by finding similarity between the variable region DNA and amino acid sequences of the 7C7:C5 Fv and the variable DNA and amino acid sequences of other antibodies, particularly in areas that code for or comprise the amino terminus. The sequences of the L_κ-V_κ-J_κ-C_κ” and L_Η-V_Η-D-J_Η-C_Η” genes were extracted from the sequences of the plasmids and, thus, the DNA sequences for the L_κ-V_κ-J_κ-C_κ” and L_Η-V_Η-D-J_Η-C_Η” genes were isolated. The amino acid sequences were used to construct molecular models of the Fv fragment of the 7C7:C5 MAb.
Homology Modeling of the 7C7:C5 Fv

Homology modeling of Fvs requires using similar \(V_\kappa\) and \(V_H\) amino acid sequences with crystallographic structures in the PDB as templates. Using Modeller9v4 required producing a command file which tells the software exactly what to model and any specialized restraints to place on the model. In this case, the automodel function of Modeller 9v4 was applied in which spatial restraints were applied based on the listed templates. The only other necessary script files are the alignment file and the corresponding PDB files for the structures mentioned in the alignment file. Amino acids of crystallographic structures used as templates were aligned by keeping not only identical but also similar residues lined up with the corresponding residues in the 7C7:C5 variable sequences. While using similar residues is not as good as using identical residues for homology modeling, using similar residues should help to produce an overall good fit for residues that are not as common among the selected templates by maintaining overall coordinate consistency with templates.

The first template (PDB #1QOK\textsuperscript{122}) listed in the alignment files was a crystallographic structure of a peptide linked murine IgG1 Fv. This template was for an entire Fv in which the \(V_H\) and \(V_\kappa\) are covalently linked in normal spatial alignment to one another using a peptide linker comprised entirely of glycine residues. The template 1QOK was selected mainly for the purpose of bringing the two variable chains within contact of each other. Listing this template first to be read in the alignment files allowed for its coordinates to be read first successfully bringing together the two chains. This template was a recombinant anti-CEA single chain Fv and showed only 52% amino acid sequence identity (65% similarity) with the 7C7:C5 Fv.
The remaining templates for each chain were selected based on PDB similarity rankings to sequences of the 7C7:C5 $V_\text{H}$ and $V_\kappa$ separately. Two separate groups of templates (crystallographic structures in unbound and antigen-bound conformations) were selected for each variable chain of the Fv. The templates of unbound and antigen-bound conformations were used to generate separate models of the 7C7:C5 Fv in consideration of the relative twists or rotations that occur in the Fv upon antigen binding, some of which can be large in size.$^{123-125}$

In addition to 1QOK, two groups of templates for modeling the $V_\kappa$ were downloaded from the PDB: unbound templates (PDB # 1E60$^{124}$, 2FAT$^{125}$, and 1AIF$^{126}$) and antigen-bound templates (PDB # 1E6J$^{124}$, 2FD6$^{127}$, and 2FBJ (Bhat, T.N., Padlan, E.A. & Davies, D.R. [To be published]). Only a few templates were chosen for modeling the 7C7:C5 $V_\kappa$ simply because the identity among these templates is relatively high. Furthermore, Modeller9v4 software collectively incorporates spatial restraints from these templates. The spatial restraints generated generally improve with higher numbers of templates used, with the exception of having an excellent or a few excellent high identity templates that resemble the targeted structure quite closely.$^{121}$

These few templates in this case are good choices for modeling the 7C7:C5 $V_\kappa$ because structures tend to be conserved among like sequences. The basis for this assumption is that high identity sequences of like proteins have many of the same building blocks in the same sequential order. This assumption is further supported for antibody variable regions in a study by Chothia $et$ $al.$$^{45}$, in which conformations for five of the six CDR loop regions are believed to be determined by some key conserved residues. Furthermore, comparisons of these loop regions showed that they have a small
repertoire of conformations, in which conserved residues lead towards one particular conformation.

The closest match to the 7C7:C5 $V_\kappa$ that has a crystal structure is the $V_\kappa$ from Fab13b5 Anti-p24 HIV-1 capsid protein (PDB # 1E6O for unbound and 1E6J for antigen-bound). The match starts with EIVLTQ, the active $V_\kappa$ amino terminus, and shares 95% identity (including RADAAP of amino terminus of $C_\kappa$). In fact, only five amino acids out of 105 amino acids in the active $V_\kappa$ are different. Another similar active $V_\kappa$ with a crystal structure is from an anti-urokinase plasminogen activator receptor (Upar) (PDB # 2FAT for unbound and 2FD6 for antigen-bound). The anti-Upar shares 93% identity with the 7C7:C5 active $V_\kappa$ plus RADAAP. Only seven amino acids out of 105 amino acids in the active $V_\kappa$ are different. These crystal structures served as a good starting point for modeling the 7C7:C5 $V_\kappa$. A crystal structure of an active $V_\kappa$ was used from an anti-idiotypic Fab (PDB # 1AIF) as an additional unbound template for part of the CDR-L3, the carboxyl terminus, and the amino terminus of the $C_\kappa$. A final active $V_\kappa$ crystal structure (sharing 93% identity with the 7C7:C5 $V_\kappa$) was used from an anti-galactan Fab (PDB # 2FBJ) for generating the antigen-bound model. Galactan is a galactose based polysaccharide and, thus, may help to model the possibility of Fv interactions with the sugar moieties of nucleic acids.

A collective approach to generating coordinates for the $V_\kappa$ was chosen, in which many $V_\kappa$ chains of crystallographic structures from murine IgG1 Fab fragments were used as templates for generating complete sets of coordinates for the unbound template and bound template $V_\kappa$ models. Generally, spatial restraints improve for a given model with increasing number of templates used. Probability density functions (pdfs) for
individual spatial restraints, such as C_α-C_α distances and main-chain dihedral angles, are combined to find the most probable coordinates for a given residue. As a result, more templates essentially equal more data to use in calculation of pdfs for each residue leading to calculated residue coordinates closer to a true value. Also, the model tends to improve in structure with higher percentage identity templates in like proteins.

The V_H was modeled based on sixteen total templates (1QOK, 1HQ4, 1F8T, 1S5I, 32C2, 1AY1, 1KCU, 3CFE, 2A0L, 1ORS, 1EZW, 1BAF, 1KEN, 1KCS, 2FON, and 1I9I) for the unbound template model and seventeen total templates (1QOK, 1ORS, 1CF8, 1F90, 1BAF, 1KCR, 1KEN, 2A0L, 1BGX, 1KC5, 1NCW, 3CFD, 1EZV, 1S5I, 1KCS, 2FON, and 1I9I) for the antigen-bound template model. The purpose of the first template was already described. The main templates used to construct the 7C7:C5 V_H were the following PDB files: 1HQ4, 1F8T, 1S5I, 32C2, 1AY1, 1KCU, and 3CFE for unbound conformation and PDB # 1ORS, 1CF8, 1F90, 1BAF, 1KCR, 1KEN, 2A0L, 1BGX, 1KC5, 1NCW, and 3CFD for antigen-bound conformation. The main unbound and antigen-bound templates both ranged from 84% to 80% identity and 91% to 86% similarity with the 7C7:C5 V_H sequence.

The remaining templates were used in both unbound and antigen-bound template V_H models in a given region, regardless of whether or not the crystal structure of the Fab was with antigen bound or not. These remaining templates for unbound and antigen-bound conformations of the V_H were used as a means of adding extra templates for refining the CDR regions of the 7C7:C5 V_H, in which only the parts of these templates that showed high identity with a certain part of the 7C7:C5 V_H were used. For the
unbound template model these templates included 2A0L, 1ORS, 1EZV, 1BAF, 1KEN, 1KCS, 2FON, and 1I9I. Of these templates 2A0L, 1ORS, 1EZV, 1BAF, and 1KEN were considered to be good templates for refining CDR-H1; 1KEN and 1KCS were considered to be good templates for refining CDR-H2; and 2FON, 1BAF, and 1I9I were considered to be good templates for refining CDR-H3.

The basis for choosing the majority of these templates was simply based on high sequence identities in a particular CDR and the framework area surrounding that CDR; however, the templates (2FON, 1BAF, and 1I9I) picked for CDR-H3 all bind to compounds that contain phenyl groups (acyl-Coenzyme A (CoA), dinitrophenyl, and testosterone, respectively). The only non-antibody template used in generating these models is 2FON, an acyl-CoA oxidase. Nevertheless, it is also the only template that contained the complete 101-WLPLAY-106 of CDR-H3 and, furthermore, it binds to acyl-CoA, of which CoA is a coenzyme that contains adenosine in a major portion of its structure. As a result, 2FON was deemed to be a rather significant template despite being non-antibody and despite being used for only a small portion of the generated antibody model.

In the bound template model, the following partial templates were used: 1EZV, 1S5I, 1KCS, 2FON, and 1I9I. By using partial (2A0L and 1ORS) and whole (1BAF and 1KEN) templates that are present in the bound template model also for the unbound template model, the two models are kept similar enough that they still resemble each other. Such integration of templates between models while allowing for unique templates to stay specific to each model, such as 3CFE (a crystal structure of purple-fluorescent Fv of Ep2-25c10) for the unbound template model and 3CFD (a crystal structure of purple-
fluorescent Fv of Ep2-25c10 in complex with stilbene hapten) for the bound template model, should provide reasonable models of the unbound Fv and the antigen-bound Fv in relation to conformational similarity with one another while still maintaining key conformational distinctions between the two models.

**Refinement of the Homology Models of the 7C7:C5 Fv**

Protonation status of residues was assessed and modified according to pKₐ using the program H++\(^{147-148}\). Salinity was set to the default, 0.15 M, which is a typical physiological salinity. The external dielectric coefficient was set to the default, 80, as a typical dielectric coefficient for saline solvents and the internal dielectric coefficient was set to 6, which is slightly higher than the default internal dielectric coefficient of 4. The purpose of raising the internal dielectric coefficient was that the binding pocket of an Fv lies near the surface as opposed to the center of the Fv, in which dielectric coefficients around 10 are more accurate for pKₐ prediction. Nevertheless, 10 was not selected as an internal dielectric coefficient because His L33 was the deepest residue in the binding pocket and, thus, lay in between the center of the Fv and the surface residues. His L33 is one of the only two residues that are polar amino acids in the binding pocket and, thus, was one of the main residues to which the pKₐ calculation was actually relevant. As a result, an intermediate internal dielectric coefficient of 6 was chosen. Poisson-Boltzmann calculations of pKₐ at a solvent pH of 7 were performed by H++ for each residue in the Fv model.

Both unbound-template and bound-template based models of the 7C7:C5 Fv were minimized by a limited memory Broyden-Fletcher-Goldfarb-Shanno (L-BFGS)
method \textsuperscript{149-150} to an RMS gradient of 0.1 kcal/mol/Å using AMBERff99 parameters \textsuperscript{151} in Tinker v. 4.2 \textsuperscript{152-153}. The L-BFGS minimization method uses a large scale unconstrained non-linear quasi-Newton approximation to converge to a gradient. The overall goal of the minimization was to leave the models as close as possible to the original applied crystallographic data that was used to generate the models, while fixing any major problems with the models such as overextended bond distances or unusual bond angles. The resulting minimized models of the 7C7:C5 Fv were converted from a XYZ format (a Tinker coordinate file format providing three-dimensional coordinates or coordinates of the X, Y, and Z planes) into a PDB format (the PDB coordinate file format providing three-dimensional coordinates accepted by various widely used modeling software) in Tinker v. 4.2

To help pinpoint unusual structural areas of the 7C7:C5 Fv models, the C\textsubscript{α} root mean square deviations (RMSDs) of the areas aligned for generation of the 7C7:C5 Fv models were calculated using DeepView/Swiss-PDB Viewer \textsuperscript{154}. The C\textsubscript{α} RMSDs were calculated using three different reference structures. The first reference structure upon which other structures (template crystal structures) were superimposed was the energy minimized model of the 7C7:C5 Fv. The second and third reference structures were the only complete Fv template (PDB #1QOK) and the most similar template in amino acid sequence to the 7C7:C5 V\textsubscript{H} or V\textsubscript{κ} (varies with model and segment), respectively.

All C\textsubscript{α} RMSDs were calculated excluding residues of the constant region. This area is incomplete in the 7C7:C5 Fv models and, thus, may have folded differently than typical during minimization. The first two residues of the 7C7:C5 V\textsubscript{H} and the first five residues of the 7C7:C5 V\textsubscript{κ} were also excluded from C\textsubscript{α} RMSD calculations. The C\textsubscript{α}
RMSDs in the amino terminus of the C_{H}1 and the amino terminus of the V_{\kappa} indicated an unnatural association between residues of these two areas since including these areas resulted in at least a two fold increase in C_{\alpha} RMSD. As a result, these areas were not included in the C_{\alpha} RMSD calculations.

The “stereochemical quality” of the 7C7:C5 Fv model was assessed by PROCHECK\textsuperscript{155-156}, a suite of programs for comparing the geometry of residues in a subject protein to stereochemical parameters for such residues derived from well-refined, high resolution crystallographic structures. Special consideration in the model was given to residues in the binding site as well as to residues of the amino terminus of the C_{H}1 and C_{\kappa}. Residues of the binding site may fluctuate from the allowed range for that residue due to conformational changes as a result of ligand binding, while C_{H}1 and C_{\kappa} N-terminus residues may fluctuate from the allowed range due to the lack of the remainder of the C_{H}1 and C_{\kappa} normally present in a Fab fragment.

The model quality assessment was performed before and after minimization using Tinker v. 4.2. The PDB file for the energy minimized model of the 7C7:C5 Fv was loaded into ADT-1.5.2\textsuperscript{157} to setup for docking studies in AutoDock-4.0\textsuperscript{77,158}. Ligands for AutoDock-4.0 were built in SpartanES-02\textsuperscript{159} using a library of nucleic acid fragments. Hydrogens were added in SpartanES-02 to complete the ligands and then the ligands were saved as PDB files and loaded into AutoDockTools (ADT)-1.5.2 to setup for docking studies in AutoDock-4.0.
Preliminary “Blind Docking” Studies of the 7C7:C5 Fv with d$_{6m}$A and Related Antigens

The general area of the paratope was predicted by a set of rigid protein “blind docking” experiments with flexible ligand using a Lamarckian Genetic Algorithm (LGA) in AutoDock-4.0. For the “blind docking” experiments with d$_{6m}$A, dA, and ssDNA A-$^6m$A-T trinucleotide as ligands, the xyz-dimensions in all of the grid boxes were set to 61 x 61 x 61, in which the total number of grid points was 226,981. The center of the Fv was at the following coordinates for all rigid Fv docking experiments: (7.765, 33.508, 9.928). A total of four, three, and one rigid Fv docking experiment(s) were performed for d$_{6m}$A, dA, and A-$^6m$A-T, respectively. All of the docking experiments were performed with wide grid boxes along the upper V$_\kappa$-V$_H$ interface (region of the Fv in the opposite direction of the constant regions that lies proximal to the midline of the Fv where the V$_\kappa$ and V$_H$ associate together).

Two docking experiments for d$_{6m}$A and two for dA used a spacing factor of 0.508 Å between grid points, in which the central coordinates of the grid boxes are the following: (4.696, 30.018, 26.196) and (9.156, 40.607, 22.156). The spacing factor was adjusted to 0.853 Å for the grid box for A-$^6m$A-T to accommodate the larger size of this antigen, in which the central coordinates of this box are (4.772, 39.569, 20.052). The spacing factor of 0.853 Å and the central grid box coordinates of (4.772, 39.569, 20.052) were also used for one docking experiment for d$_{6m}$A and dA separately. One additional docking experiment was performed for d$_{6m}$A using a spacing factor of 0.375 Å and the central grid box coordinates of (2.027, 36.135, 27.448). The grid boxes with the spacing of 0.508 Å and the central coordinates of (4.696, 30.018, 26.196) resulted in the highest energy binding modes for d$_{6m}$A and dA. These conformations were selected for further
analysis. A binding mode for A-\textsuperscript{6m}A-T that resembled the selected binding modes of \textsuperscript{6m}A and dA was found using the grid box spacing of 0.853 Å and the central coordinates of (4.772, 39.569, 20.052).

The docking parameter file for these three ligands was set for a population of 150 individual ligand binding modes, a maximum of 2.5 x 10\textsuperscript{6} energy evaluations, a maximum of 2.7 x 10\textsuperscript{4} generations, a mutation rate of 2\%, and a crossover rate of 80\%. The probability of performing a local search on an individual ligand mode was set to 6\%. The number of individual modes that proceed to the next generation was set to 1 and the number of generations analyzed to determine the threshold least favorable conformation in the population was set to 10. The local search algorithm was set for 300 iterations with a lower bound step size of 0.01. Four successive successes or failures were required for lower bound step size to change. A total of 10 LGA trial runs were performed.

Ligand torsional degrees of freedom were set to 4 for \textsuperscript{6m}A and dA and 15 for A-\textsuperscript{6m}A-T. Torsional degrees of freedom were set for the fewest active atoms. The torsional coefficient was set to 0.274 for each of the ligands. The translation step, quaternion step, and torsion step were set to 2 steps/Å, 50 steps/degree, and 50 steps/degree, respectively, for each of the ligands. All results were clustered according to rmsd and ranked according to total binding energy (the sum of the intermolecular energy, the internal energy, the unbound system’s or starting state energy, and the torsional free-energy penalty) represented as \(\Delta G\). All grid and docking parameter files were prepared using ADT-1.5.2 with the corresponding tutorial \textsuperscript{157} as a guide. A dissociation constant (\(K_d\)) was calculated for each viewable docking mode using the equation
\[ K_d = \exp((\Delta G \times 1000 \text{ cal/kcal})/(RT)) \] where \( \Delta G \) is the binding energy in kcal/mol, \( R \) is the gas constant 1.98719 cal\,\text{mol}^{-1}\text{K}^{-1}, and \( T \) is 298.15 K.

**Refined Docking Studies with Flexible Protein Residues**

Further docking experiments using AutoDock-4.0 involved binding of the antigens \( d^{6m}A, dA, \) and \( A^{6m}A-T \), in which five residues in the area of the binding pocket were selected to be flexible (Tyr H104, Tyr L35, Ile L46, Tyr L48, and Ser L55). In addition, grid boxes and certain docking parameters were adjusted for a more refined analysis. The Fv with five flexible residues was centered at (7.824, 33.593, 9.791) for all flexible binding pocket docking experiments. All grid boxes for the flexible binding pocket docking experiments were centered around the binding pocket, according to the site of docking of \( d^{6m}A, dA, \) and \( A^{6m}A-T \) from selected binding modes found in rigid Fv docking experiments.

Grid boxes for \( d^{6m}A \) and \( dA \) were set to xyz-dimensions 51 x 51 x 51, in which the total number of grid points was 132,651. The grid boxes for \( d^{6m}A \) and \( dA \) were centered at (0.022, 29.553, 20.207). The grid box for \( A^{6m}A-T \) was set to xyz-dimensions 71 x 75 x 57, in which the total number of grid points was 303,525. The grid box for \( A^{6m}A-T \) was centered at (0.075, 29.19, 20.95). The spacing factor between grid points was set to 0.375 Å for \( d^{6m}A \) and \( dA \) and 0.392 Å for \( A^{6m}A-T \).

Adjusted docking parameters include a maximum of \( 12.5 \times 10^6 \) energy evaluations and a maximum of \( 7.5 \times 10^4 \) generations for \( d^{6m}A \) and \( dA \). A maximum of \( 15 \times 10^6 \) energy evaluations and a maximum of \( 1.0 \times 10^5 \) generations were set for \( A^{6m}A-T \). A total of 25, 50, and 50 LGA trial runs were performed for \( d^{6m}A, dA, \) and \( A^{6m}A-T, \)
respectively. The number of active torsions were set to 16 for $d^{6m}A$ and $dA$ and 27 for $A^{6m}A-T$. Ligand torsional degrees of freedom were set to 4 for $d^{6m}A$ and $dA$ and 15 for $A^{6m}A-T$, in which the fewest active atoms were selected for torsions. All other parameters were kept the same as the parameters used for the preliminary “blind docking” experiments. A $K_d$ was calculated for each viewable binding mode. Changes in dihedral angles and atom distances were measured for each of the five flexible residues in the selected binding modes using ADT-1.5.2, in which the corresponding rigid residues served as initial conformations. The five flexible Fv residues in each of the selected binding modes were superimposed on the corresponding rigid Fv residues to find a relative axis of measurement and to visually inspect all calculated angles.
RESULTS

Blotting Results for the Detection of d$_{6m}$A in DNA with the 7C7:C5 MAb

Results of two dot blot experiments and one optimized slot blot experiment were used to compare binding of the 7C7:C5 MAb to single-stranded DNA containing a single deoxy-N$^6$-methyladenosine (d$_{6m}$A) and to unmethylated DNA. The comparison essentially demonstrated the affinity difference between the 7C7:C5 MAb against a single d$_{6m}$A versus a single deoxyadenosine (dA) in DNA. All methylated DNA used in the blotting studies was methylated with EcoRI methylase. The first dot blot was for 1:10 dilutions of methylated DNA versus unmethylated DNA from 0.1 ng to 1 µg, in which a dark purple dot at 0.1 µg of methylated DNA was detected and a faint, circular purple outline at 1 µg of unmethylated DNA was detected. No other purple stains were detected for this blot.

The second dot blot compared treatment with the 7C7:C5 MAb to treatment without the 7C7:C5 MAb for the following dilutions of methylated and unmethylated DNA: 0.0625 µg, 0.1 µg, 0.125 µg, 0.25 µg, 0.5 µg, 0.75 µg, and 1 µg. One membrane was treated with 7C7:C5 MAb/RPMI/10% FCS and the other membrane was treated with RPMI/10% FCS. The membrane with 7C7:C5 MAb treatment showed dark purple dots for methylated DNA at 0.25 µg to 0.1 µg and only purple, circular outlines for unmethylated DNA of which the most intense was at 1 µg. The membrane without 7C7:C5 MAb treatment showed only one or two extremely faint purple outlines for unmethylated DNA.
As a result, the 7C7:C5 MAb is clearly the ingredient in the 7C7:C5 MAb/RPMI/10% FCS medium that is recognizing methylated and unmethylated DNA. A barely visible background may be due to RPMI/10% FCS medium or simply the staining process. Although treatment with RPMI/10% FCS resulted in one or two barely visible purple outlines for unmethylated DNA, it did not result in any staining for methylated DNA. This result also suggests the possibility that some ingredient in RPMI/10% FCS may be binding only to unmethylated DNA with low affinity or in low amount. This ingredient must be some molecule that the anti-mouse IgG antibody shows cross-reactivity for, even if it is only with extremely low affinity.

Slot blots are more reliably quantifiable than the dot blots due to the use of the S&S Minifold Slot Blot Filtration Apparatus. Results from a protection assay provided evidence that the methylated DNA used in the optimized slot blot was indeed methylated (Figure 6).
Figure 6. The protection assay for verifying methylation of DNA used in the optimized slot blot. Negative control (unmethylated pUC-19 without EcoRI RE treatment) is in the left lane, positive control (unmethylated pUC-19 with EcoRI RE treatment) is in the middle lane, and pUC-19 methylated with M.EcoRI and treated with EcoRI RE is in the right lane. A 10 µL aliquot of each DNA sample was mixed with a final 1X loading dye and the samples were then loaded onto a 1% agarose, ≥0.1 µg/mL EtBr gel. The gel was electrophoresed at 72 V for approximately two hours. Picture of the gel was taken by the UV Kodak 120 Camera with two seconds of UV exposure.

The optimized slot blot was performed for the following dilutions of methylated and unmethylated DNA: 0.6 ng, 0.9 ng, 4.5 ng, 9 ng, 45 ng, 90 ng, 0.18 µg, 0.45 µg, 0.9 µg, and 1.8 µg. Unmethylated DNA at a dilution of 1.8 µg and 0.9 µg showed not only the most intense stains on the blot, but also showed stains with the largest areas. The stain at 1.8 µg of unmethylated DNA showed a 1.6 fold greater intensity and a considerably greater stain area than the stain at 0.9 µg of unmethylated DNA. No band detection was noticed at 1.8 µg of methylated DNA. Methylated DNA at a dilution of 0.9 µg showed a 4.5 fold lower intensity and a smaller stain area than unmethylated DNA at a dilution of 0.9 µg. These results have actually been seen in a similar manner in the first
and second dot blots since the highest concentrations of methylated DNA on these blots did not give the most intense dot stains for methylated DNA.

Although these results may seem to contrast the notion that the 7C7:C5 MAb is specific for DNA containing d$^6$m A, they can be explained in such a manner that further supports that the 7C7:C5 MAb is specific for DNA containing d$^6$m A. The amount of 7C7:C5 MAb that binds to methylated DNA at 1.8 µg and 0.9 µg is too much biomass for the membrane to hold onto. Additionally, the amount of amIgG-AP that in turn binds to the 7C7:C5 MAb also contributes biomass. As a result, the DNA-antibody complex breaks free at some point after the addition of the 7C7:C5 MAb when the biomass becomes too great for the membrane to hold and is pulled by the accumulated mass of the antibody into solution. Characteristically, at 2 µg of DNA, antibodies only with high affinity (K$_d$ lower than 10$^{-8}$ M) for the DNA antigen can break the covalent linkage of the bound DNA to the nitrocellulose and pull the DNA into solution$^{43}$.

Simply, the lack of appearance and low appearance of stain on the membrane at methylated DNA dilutions of 1.8 and 0.9 µg, respectively, is a result of too much binding (too high binding affinity) rather than too little binding (too low binding affinity). This explanation is supported by the appearance of substrate solution turning purple in areas around the 1.8 and 0.9 µg dilutions of methylated DNA, in which the stain did not stick to the membrane. An opposite trend, however, starts to occur at 0.45 µg of methylated DNA because the biomass of the DNA+antibody+anti-antibody+substrate complex is low enough for the membrane to hold onto a good portion of the complex. At 0.45 µg of methylated DNA, the intensity was 1.8 fold greater than the intensity at 0.45 µg of
unmethylated DNA. Also, the area at 0.45 µg of methylated DNA was larger than the area for 0.45 µg of unmethylated DNA.

At 0.18 µg of methylated DNA, the intensity was 5.7 fold greater than the intensity at 0.18 µg of unmethylated DNA. Plus, the area at 0.18 µg of methylated DNA was larger than the area for 0.18 µg of unmethylated DNA. Methylated DNA at a dilution of 0.09 µg showed a 3.1 fold greater intensity than unmethylated DNA at a dilution of 0.18 µg. No detectable stain appeared at 0.09 µg of unmethylated DNA.

These results suggest that the 7C7:C5 MAb has a binding affinity for DNA containing a single d\textsuperscript{6m}A that is approximately six fold greater than the binding affinity it has for unmethylated DNA. Results for the optimized slot blot are shown in Figure 7 and listed in Table 4.
Figure 7. Two pictures of the results from the optimized slot blot under different lighting. Pictures were taken of a section of the nitrocellulose membrane where bands appeared. Pictures were taken by (a) a digital camera under a desk lamp for lighting and (b) a Kodak DS UV Camera with two seconds of UV exposure. The membrane was inverted for the picture in (b). The picture in (b) was used for measurement of band intensities.

Table 4. Slot blot band intensities comparing binding of the 7C7:C5 MAb to M.EcoRI methylated pUC-19 DNA versus unmethylated pUC-19 DNA

<table>
<thead>
<tr>
<th>Amount of DNA (µg)</th>
<th>Unmethylated DNA</th>
<th>Methylated DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.8</td>
<td>287.64</td>
<td>40.31</td>
</tr>
<tr>
<td>0.9</td>
<td>180.72</td>
<td>76.94</td>
</tr>
<tr>
<td>0.45</td>
<td>42.24</td>
<td>44.13</td>
</tr>
<tr>
<td>0.18</td>
<td>7.7143</td>
<td>24</td>
</tr>
<tr>
<td>0.09</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*Band intensities were measured by using Kodak DS UV software. The intensity of each band was measured from left center of the band to middle center of the band. Equal areas of measurement were used for each band intensity measurement.
Amplification of cDNA for the 7C7:C5 V\textsubscript{\kappa} and V\textsubscript{H} Genes

The 7C7:C5 V\textsubscript{\kappa} and V\textsubscript{H} genes were successfully synthesized and amplified by performing RT-PCR on RNA isolated from thawed 7C7:C5 hybridoma cells that were previously frozen at \(\geq -70^\circ\text{C}\). Figure 8a shows a 1% agarose, 0.1 \(\mu\text{g/mL}\) EtBr gel prepared as described in the Material and Methods section with successful 7C7:C5 total RNA isolation. The isolation of RNA was performed as described in the Material and Methods section using TRIzol/chloroform separation of phases. The result shown in Figure 8a suggested that enough RNA was isolated for cDNA synthesis and amplification, respectively. The V\textsubscript{\kappa} gene was successfully amplified with this RNA. The result shown in Figure 8b suggested that only trace levels of RNA led to the successful amplification of the V\textsubscript{H} gene. In each RNA isolation using the TRIzol method, DNA contamination was produced and, thus, this co-isolate appears to be inherent to the method. This DNA contamination did not cause a problem for cDNA synthesis.

Figure 8. Total RNA isolation used for cDNA synthesis of the (a) V\textsubscript{\kappa} and (b) V\textsubscript{H} genes. Isolation of total RNA was done on thawed 7C7:C5 hybridoma cells using the TRIzol method as described in the Material and Methods. A 5 \(\mu\text{L}\) aliquot of RNA from each RNA sample was mixed with a final 1X bromophenol blue loading dye and loaded onto a 1% agarose, 0.1 \(\mu\text{g/mL}\) EtBr gel. The gels were electrophoresed at 72 V for forty minutes. Gels were analyzed by UV exposure.
The 7C7:C5 V\textsubscript{κ} and V\textsubscript{H} cDNAs were synthesized and amplified using RT-PCR. Both the 7C7:C5 V\textsubscript{κ} and V\textsubscript{H} cDNAs were reamplified by PCR to produce more of each and simply to repeat the experiment to confirm the first result. Figures 9 and 10 show results of the amplification of the 7C7:C5 V\textsubscript{κ} and V\textsubscript{H} genes, respectively. The RT-PCR was performed as described in the Materials and Methods section. The primers used are listed in Tables 2 and 3.

![Figure 9](image1.png)

**Figure 9.** (a) Amplification of the 7C7:C5 V\textsubscript{κ} gene and (b) measurement of the V\textsubscript{κ} gene amplicons. cDNA synthesis and amplification were performed according to standardized RT-PCR protocol listed in the Materials and Methods. For (a) and (b), a 10 µL aliquot of each PCR product mixed with a 1X loading dye was loaded into separate lanes on a 1% agarose, 0.1 µg/mL EtBr gel and electrophoresed at 59 V for seventy minutes. For (b), 8 µL of a DNA ladder is in Lane 1. Gels were analyzed by UV exposure.
Figure 10. Amplification and measurement of the 7C7:C5 V\textsubscript{H} gene. cDNA synthesis and amplification were performed using standardized RT-PCR protocol found in the Material and Methods. Aliquots of 10 \( \mu \)L of each PCR product mixed with a final 1X loading dye were electrophoresed on a 1\% agarose, \( \geq 0.1 \) \( \mu \)g/mL EtBr gel at 59 V for seventy minutes in top lanes 3-10. PCR products were distributed as follows, according to L\textsubscript{H} 5’ primer name: APg1AZ, AVg1XM, APg1X, APg1AM, APg1AMU, APg1KEA, VH1, and VH2 in lanes 3-10 respectively. Lane 1 has 8 \( \mu \)L of a DNA ladder. Gels were analyzed by UV exposure.

In the case of the V\textsubscript{\kappa} cDNA, two 5’ primers (MLALT1 and MLALT2) and the 3’ CkFORa resulted in amplicons of appropriate size at \( \sim 426 \) bp. Amplification with MLALT1 produced a much fainter band with more primer dimers than MLALT2. In turn, MLALT2 gave a much cleaner PCR product. Reamplification of the MLALT1 and 2 PCR products produced a noticeable band at \( \sim 426 \) bp only for the MLALT2 PCR product (Figure 11a). Also, primer dimers seemed even more abundant for the reamplification with MLALT1 than the original amplification with MLALT1, while reamplification with MLALT2 still maintained relatively low amount of primer dimers despite being slightly fainter than the original amplification with MLALT2. The primers that gave successful amplification of the V\textsubscript{H} gene were the 5’ APg1AZ and the 3’
Cg1FORa. The \( V_H \) gene appeared at \(~432 \) bp. The reamplification of the \( V_H \) gene with APg1AZ and Cg1FORa produced a band at least as intense as the original amplification if not more (Figure 11b).

Figure 11. (a) Reamplification of 7C7:C5 (a) \( V_\kappa \) and (b) \( V_H \) PCR products. An aliquot of 2 \( \mu \)L of PCR product was reamplified using standardized PCR protocol listed in the Materials and Methods. A 10 \( \mu \)L aliquot of each reamplified PCR product mixed with a final 1X loading dye was electrophoresed at 59 V for seventy minutes on a 1% agarose, \( \geq 0.1 \mu g/mL \) EtBr gel. Gels were analyzed by UV exposure.

Why were two PCR products of the same size produced using two distinct primers? The likely reason is that an inherent gene for the aberrant kappa chain is present in the 7C7:C5 hybridoma cells. The aberrant kappa chain gene is known to be produced by hybridomas from the murine SP2/0 MOPC21 cell line (the cell line from which the 7C7:C5 hybridoma originates) along with the functional kappa chain gene\(^{118-119}\). One of the 5’ primers may be amplifying the aberrant \( V_\kappa \) gene, while the other 5’ primer is
amplifying the 7C7:C5 Vκ gene. The desired DNA sequence is of course the one for the active Vκ gene and not for the inactive, aberrant Vκ gene.

Upon performing a sequence alignment using LALIGN\textsuperscript{160} in ExPASy, MLALT1 shared 96% identity with the SP2/0 aberrant Vκ sequence and, thus, is quite likely to anneal to the aberrant Vκ sequence; while MLALT2 shares only 42% identity with the SP2/0 aberrant Vκ sequence and, thus, is unlikely to anneal to the aberrant Vκ sequence. Annealing of the forward and reverse primers is essential to the success of the amplification during PCR. Thus, the most likely possibility is that MLALT2 annealed to the 7C7:C5 Vκ cDNA resulting in its amplification.

Furthermore, the sequence for the SP2/0 aberrant Vκ gene was checked using NEBcutter\textsuperscript{161} v.2.0 and does not contain a KpnI restriction site, a fairly common restriction site among murine Vκ sequences. Looking at Figure 12, the 7C7:C5 MLALT2 amplicon was successfully digested with KpnI and, thus, must contain a KpnI restriction site. The presence of a KpnI site along with alignment results suggested that the 7C7:C5 MLALT2 amplicon is indeed the 7C7:C5 Vκ sequence and not the SP2/0 aberrant Vκ sequence. Fortunately, no additional test was needed for the obtained VH gene since the majority of hybridomas only produce the functional heavy chain transcript.
Cloning of the 7C7:C5 \( V_\kappa \) and \( V_H \) Amplicons

The 7C7:C5 \( V_\kappa \) and \( V_H \) amplicons were then cloned into two separate pJet1/Blunt vectors using methods described in the Material and Methods. The purpose of cloning is 1) to produce enough DNA for sequencing and 2) to make sequencing of small PCR products more feasible by incorporating them into a plasmid. This plasmid was used to transform chemically competent JM109 \( E.coli \). The plasmid amount replicated along with the replicating transformed \( E.coli \).

After growing the transformed \( E.coli \) overnight, the plasmid DNA was isolated from the rest of the cell contents by GeneJet mini-prep. A kit proved necessary for successful isolation of these plasmids, as the method described for lab bench mini-prep
used for producing plasmid DNA for dot/slot blots in the Materials and Methods section did not function well for isolation of the 7C7:C5 V<sub>K</sub> and V<sub>H</sub> plasmids (plasmids containing either the 7C7:C5 V<sub>K</sub> or V<sub>H</sub> PCR product insert). In addition, despite using a kit for isolation of the 7C7:C5 V<sub>K</sub> and V<sub>H</sub> plasmids, the plasmids often required further purification by phenol/chloroform/isoamyl alcohol extraction followed by precipitation of DNA in the aqueous phase as described in the Material and Methods section before submitting samples to the University of Michigan for DNA sequencing. Figure 13 shows an example of a 7C7:C5 V<sub>K</sub> purified plasmid.

![Purified 7C7:C5 V<sub>K</sub> Plasmid](image)

Figure 13. Purification and precipitation of a 7C7:C5 V<sub>K</sub> plasmid. A 5 µL aliquot of purified and precipitated plasmid was mixed with a final 1X loading dye and electrophoresed at 72 V for one hour on a 1% agarose, 0.1 µg/mL EtBr gel.

Generally, isolation of plasmid was performed on more than one culture of transformed <i>E.coli</i>, but plasmid isolated from each culture was kept separate so as to not mix products from separate sets of growth. Mixing plasmid products can lead to two different plasmids in one mix and, as a result, both plasmids may become unable to be sequenced. DNA cannot be separated from DNA, especially like DNA (same vector but different inserts), so easily, in which the best case was to avoid this problem.
A double digestion experiment using Fast Digest (FD) \textit{XhoI} and FD \textit{XbaI} restriction endonucleases was performed to demonstrate that an appropriately sized insert was cloned into the pJet1/blunt vector before submitting samples for sequencing. In this case, the insert was $\sim 451$ bp (426 bp of PCR product plus 25 bp of vector) for the 7C7:C5 $V_\kappa$ excised fragment and $\sim 457$ bp (432 bp of PCR product plus 25 bp of vector) for the 7C7:C5 $V_\lambda$ excised fragment. Figure 14 shows a double digestion result for two separately isolated 7C7:C5 $V_\kappa$ plasmids from two separate colonies at different locations along the same plate of growth. Each of these plasmids shows an excised fragment close to the expected size, but the bands for these fragments are faint. The band from the 7C7:C5 $V_\kappa$ Plasmid #1 (P1) was stronger than the band from 7C7:C5 $V_\kappa$ Plasmid #2 (P2).

![Image of gel electrophoresis](image)

**Figure 14.** Double digestion with FD \textit{XhoI} and \textit{XbaI} to excise the 7C7:C5 $V_\kappa$ DNA insert fragment from isolated 7C7:C5 $V_\kappa$ plasmids. An 8 \( \mu \text{L} \) aliquot of $V_\kappa$ plasmid DNA was double digested as described in the Materials and Methods. Samples were mixed with a final 1X loading dye and electrophoresed at 59 V for one hour on a 1\% agarose, 0.1 \( \mu \text{g/mL} \) EtBr gel. The excised fragments for the $V_\kappa$ inserts and the (+)-control insert are circled in orange.
Both $V_\kappa$ P1 and P2 were tested for the presence of a $KpnI$ site by digesting with $KpnI$ restriction endonuclease. The pJet1/blunt vector does not contain a $KpnI$ site, so that linearization of the plasmid represents the presence of the 7C7:C5 $V_\kappa$ gene inserted into the plasmid. The 7C7:C5 $V_\kappa$ sequence was demonstrated to possess a $KpnI$ site previously by digestion of the 7C7:C5 $V_\kappa$ PCR product with $KpnI$ RE. Figure 15 shows that the 7C7:C5 $V_\kappa$ P1 was successfully linearized by digestion with $KpnI$ RE. The $KpnI$ digestion result for the $V_\kappa$ P2 (data not shown) appeared to be identical to that for $V_\kappa$ P1. Unfortunately, not enough 7C7:C5 $V_\kappa$ P1 and P2 remained for sequencing by the end of all the analysis of these plasmids.

Figure 15. Digestion of the 7C7:C5 $V_\kappa$ P1 with $KpnI$ RE. An aliquot of 8 µL of plasmid was digested with $KpnI$ RE as described in the Materials and Methods. Samples were loaded with a final 1X loading dye onto a 1% agarose, 0.1 µg/mL EtBr gel. This gel was electrophoresed at 72 V for one hour and analyzed by UV exposure. Lane 9 (left) has 1st batch $V_\kappa$ P1 plasmid digested with $KpnI$ RE and Lane 10 (right) has 1st batch undigested $V_\kappa$ P1 plasmid.

Successful sequencing of the 7C7:C5 $V_\kappa$ and $V_H$ genes required producing numerous plasmids. The purpose of producing numerous 7C7:C5 $V_\kappa$ and $V_H$ plasmids was to compensate for various preparation problems and difficulties involved in the
sequencing of these plasmids. Due to the lack of $V_\kappa$ P1 and P2 that remained, an adjacent colony to the colony that produced $V_\kappa$ P1 had been transferred to another LBA plate and cultured on that plate. The isolated plasmid (7C7:C5 $V_\kappa$ Plasmid #3) from this colony was the first $V_\kappa$ plasmid to be sequenced, in which one of the most informative $V_\kappa$ sequencing results were obtained.

Figure 16 shows a double digestion of 7C7:C5 $V_\kappa$ Plasmid #* (P*), in which only a partial 7C7:C5 $V_\kappa$ amplicon was excised from the pJet1/blunt vector. The 7C7:C5 $V_\kappa$ P* is a plasmid that was produced from a separate cloning reaction than $V_\kappa$ P1 or P2. The $V_\kappa$ P* resulted in highly informative sequencing results for approximately the first two-thirds of the $V_\kappa$ DNA sequence.

Figure 16. Double digestion of the 7C7:C5 $V_\kappa$ Partial Insert Plasmid (P*) using FD $XhoI$ and FD $XbaI$. Aliquots of 5 µL of plasmid DNA samples were used for each digestion in each lane. Samples were loaded with a final 1X loading dye onto a 1% agarose, 0.1 µg/mL EtBr gel. This gel was electrophoresed at 59 V for one hour and analyzed by UV exposure. The band for the partially degraded $V_\kappa$ insert is circled in black in Lane 3.
Figure 17 shows results of a double digestion of 7C7:C5 V_κ P3 (a, b, and c) plasmids produced from a separate isolation of plasmid than the original 7C7:C5 V_κ P3. They are named the same merely because the same purified plasmid was stored at -20°C and then used to transform more JM109 E.coli. Two of the plasmids were prepped from transformed colonies cultured on two separate plates (Plate A and B) and the third plasmid on a third plate (Plate C) was prepped from a fresh growth of a colony from Plate A. As a result, the plasmids are essentially the same excluding possible mutations that may occur from one transformation to the next. The V_κ P3a and P3c were sequenced, assisting in a final determination of any controversial nucleotides in the V_κ DNA sequence. The double digestion experiment in Figure 17 used higher volumes of plasmid than used in other double digestion experiments, simply to produce a more intense band for the excised 7C7:C5 V_κ DNA insert.
Figure 17. Double digestion of 7C7:C5 V\(_\kappa\) P3 (a, b, and c) plasmids with FD XhoI and FD XbaI. Lane 2 contains reamplified V\(_\kappa\) PCR product. Lanes 3-6 contain undigested, XhoI digested, XbaI digested, and double digested 7C7:C5V\(_\kappa\) P3a plasmid from Plate A/Prep A, respectively. Lanes 7-8 contain undigested and double digested 7C7:C5 V\(_\kappa\) P3b plasmid from Plate B/Prep B, respectively. Lanes 9-10 contain undigested and double digested 7C7:C5 V\(_\kappa\) P3c plasmid from Plate C/Prep A, respectively. Double digestions using 20 \(\mu\)L of plasmid DNA were performed as described in the Materials and Methods. Samples were loaded with a final 1X loading dye onto a 1% agarose, 0.1 \(\mu\)g/mL EtBr gel. This gel was electrophoresed at 59 V for one hour and analyzed by UV exposure. The bands for the V\(_\kappa\) inserts are circled in black in Lanes 6, 8, and 10.

The 7C7:C5 V\(_H\) plasmids were not only difficult to isolate in sufficient concentration from JM109 E.coli transformed with them, but also these plasmids in particular generally suffered from higher impurity than the V\(_\kappa\) plasmids after GeneJet mini-prep. As a result, the strategy of using a purified plasmid from an original isolation to transform more JM109 was incorporated. The 2\(^{nd}\) generation of transformed JM109 generally grew more efficiently than the original transformation and also resulted in better isolation of plasmid (higher concentration and purity). Furthermore, the colonies

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produced from this 2nd generation were likely to exclusively contain the desired plasmid, diminishing the possibility of picking out a colony that may not contain the desired plasmid for isolation. Figure 18 shows a double digestion result for two separately isolated 7C7:C5 V\textsubscript{H} plasmids. Only the 7C7:C5 V\textsubscript{H} Plasmid #2 (P2) shows an excised fragment close to the expected size. The V\textsubscript{H} P2 was sequenced in duplicate, in which both sets of sequencing gave identical high quality DNA sequences for the V\textsubscript{H}.

![Double digestion of the 7C7:C5 V\textsubscript{H} Plasmids with FD XhoI and FD XbaI.](image)

Figure 18. Double digestion of the 7C7:C5 V\textsubscript{H} Plasmids with FD XhoI and FD XbaI. Each sample had a final 1X loading dye and was loaded onto a 1.5% agarose, 0.1 µg/mL EtBr gel in the respective order: Lane 1, DNA ladder; Lane 2, empty; Lane 3, undigested 7C7:C5 V\textsubscript{H} P1 plasmid; Lane 4, 7C7:C5 V\textsubscript{H} P1 plasmid digested with XhoI; Lane 5, 7C7:C5 V\textsubscript{H} P1 plasmid digested with XbaI; Lane 6, double digested 7C7:C5 V\textsubscript{H} P1 plasmid; Lane 7, undigested 7C7:C5 V\textsubscript{H} P2 plasmid; Lane 8, 7C7:C5 V\textsubscript{H} P2 plasmid digested with XhoI; Lane 9, 7C7:C5 V\textsubscript{H} P2 plasmid digested with XbaI; and Lane 10, double digested 7C7:C5 V\textsubscript{H} P2 plasmid. This gel was electrophoresed at 59 V for one hour and analyzed by UV exposure. An excised V\textsubscript{H} insert from the 7C7:C5 V\textsubscript{H} P2 is circled in black in Lane 10.
Obtaining the DNA Sequences for the 7C7:C5 $\kappa$ and $\text{V}_H$

Sequencing results obtained from the University of Michigan DNA Sequencing Core were further analyzed for errors and missing nucleotides. The $\kappa$ sequences commonly showed errors and missing nucleotides. The overall process of isolating a plasmid containing the 7C7:C5 $\kappa$ gene insert along with additional purification steps was repeated for numerous sequencing attempts, of which eight provided useful sequencing results. Six of the eight significant $\kappa$ sequencing results were all plasmid isolates derived from the $\kappa$ P3 isolate that contained the 7C7:C5 $\kappa$ gene insert. A separate cloning reaction was done to produce the 7C7:C5 $\kappa$ P*, in which this plasmid actually contained only a partial/degraded 7C7:C5 $\kappa$ gene.

The reported eight sequencing results for the 7C7:C5 $\kappa$ gene and corresponding chromatogram analyses were used to determine the complete 7C7:C5 $\kappa$ DNA sequence. The nucleotide sequence for the 7C7:C5 $\kappa$ from residue -16 to 54 was primarily obtained from the reverse sequencing reaction results for the 7C7:C5 $\kappa$ P* and is in congruence with four other informative sequencing results for the $\kappa$. The latter half of the $\kappa$ sequence was first primarily constructed from the forward and reverse sequencing results for the 7C7:C5 $\kappa$ P3, and then from 7C7:C5 $\kappa$ P3a and c forward and reverse sequencing results. The forward sequencing results were overall more informative for this latter half of the $\kappa$ sequence. However, the reverse sequencing result for the 7C7:C5 $\kappa$ P3c decisively showed that residue 86 was in fact a tyrosine. In addition, this same sequencing result showed that residue 105 was in fact a lysine. The 7C7:C5 $\kappa$ sequence is accurate through extensive analysis of the chromatograms along with
comparison of the various sequencing results. The 7C7:C5 V<sub>H</sub> P2, on the other hand, gave sequencing results without any errors or missing nucleotides and, thus, the only difficulties with obtaining the V<sub>H</sub> sequence were in obtaining a plasmid in high enough amount, concentration, and purity for sequencing. The complete DNA sequences and amino acid translations for the 7C7:C5 V<sub>κ</sub> and V<sub>H</sub> are reported in Figures 19 and 20, respectively.

Figure 19. The complete 7C7:C5 V<sub>κ</sub> DNA sequence and its amino acid translation. Each amino acid is placed directly below the middle of its codon and the corresponding amino acid number is directly below the amino acid. The active 7C7:C5 V<sub>κ</sub> is 105 amino acids long. Residues -16 to -1 are residues of the L<sub>κ</sub> and, thus, are not part of the active V<sub>κ</sub>. Residues 106 to 111 are residues of the amino terminus of the C<sub>κ</sub>. Underlined in bold are the three kappa chain CDRs in sequential order from CDR-L1 to CDR-L3. Nucleotides and amino acids in red or grey required analysis of the chromatograms and/or comparison of the obtained sequencing results.
Figure 20. The complete 7C7:C5 V<sub>H</sub> DNA sequence and its amino acid translation. Each amino acid is placed directly below the middle of its codon and the corresponding amino acid number is directly below the amino acid. The active 7C7:C5 V<sub>H</sub> is 117 amino acids long. Residues -13 to -1 are residues of the L<sub>H</sub> and, thus, are not part of the active V<sub>H</sub>. Residues 118 to 122 are residues of the amino terminus of the C<sub>H</sub>1. Underlined in bold are the three gamma 1 chain CDRs in sequential order from CDR-H1 to CDR-H3.

Sequence Blast Results for the 7C7:C5 V<sub>κ</sub>

Commonly, murine IgG1s seem to be a combination of sequences from Chromosome 6 for the V<sub>κ</sub> sequence and a combination of sequences from Chromosome 12 for the V<sub>H</sub> sequence. Coincidentally, the 7C7:C5 V<sub>κ</sub> DNA sequence appears to be a combination of genomic sequences from Chromosome 6. Nucleotides 36 to 326 of the 7C7:C5 V<sub>κ</sub> DNA sequence shares 98% identity with nucleotides 21173662 to 21173372.
of strain C57BL/6J Mus musculus Chromosome 6 (ref|NT_039353.7|Mm6_39393_37).

Nucleotides 1 to 35 of the 7C7:C5 Vκ DNA sequence share 100% identity with
nucleotides 21070652 to 21070618 of the previously mentioned strain C57BL/6J Mus
musculus Chromosome 6. Nucleotides 327 to 365 of the 7C7:C5 Vκ DNA sequence
share 97% identity with nucleotides 22985873 to 22985910 of the previously mentioned
assembly of Mus musculus Chromosome 6. Nucleotides 366-384, primarily Cκ” codons,
of the 7C7:C5 kappa DNA sequence share 100% identity with nucleotides 22989391 to
22989407 of the previously mentioned assembly of Mus musculus Chromosome 6. As a
result, a majority of the 7C7:C5 Vκ DNA sequence seems to have originated from
different parts of one assembly of Chromosome 6.

In addition, much of the 7C7:C5 Vκ DNA sequence (including the Lκ) showed
high similarity to predicted Mus musculus Vκ DNA sequences such as
ref|XM_001476590.1| (88% identity from nucleotide 1 to 339 of the 7C7:C5 Vκ),
ref|XM_001476823.1| (87% identity from nucleotide 1 to 311 of the 7C7:C5 Vκ), and
ref|XM_001477017.1| (87% identity from nucleotide 2 to 308 of the 7C7:C5 Vκ).
These results support that the 7C7:C5 Vκ is a Vκ that has not been previously sequenced.
The amino acid sequence, however, reveals the primary structure of the antibody. The
highest similarity in amino acid sequence viewed was 94% identity with
gb|ABC86056.1| through all 127 amino acids of the obtained 7C7:C5 kappa sequence.
This amino acid sequence and others were not very useful for modeling because no
crystal structures are available for them.

An IgBlast was done to align known Vκ sequences with the 7C7:C5 Vκ
sequence and, in turn, identify the positions of the CDRs in the Vκ sequence. Starting
with EIVLTQ, amino acids 1-23 are part of FR-L1, amino acids 24-33 are part of CDR-L1, amino acids 34-48 are part of FR-L2, amino acids 49-55 are part of CDR-L2, and amino acids 56-87 are part of FR-L3. The remaining CDR-L3, residues 88-95, and FR-L4, residues 96-105, were identified by a combination of Kabat numbering and comparative analysis with other similar \( V_\kappa \) sequences since the IgBlast does not clearly indicate these regions.

**Sequence Blast Results for the 7C7:C5 \( V_\text{H} \)**

The 7C7:C5 \( V_\text{H} \) DNA sequence appears to be a combination of genomic sequences from Chromosome 12. Nucleotides 28 to 333 of the 7C7:C5 \( V_\text{H} \) DNA sequence shares 96% identity with nucleotides 26479777 to 26479472 of strain C57BL/6J *Mus musculus* Chromosome 12 (ref|NT_166318.1|Mm12_163492_37). Nucleotides 2 to 14 and 15 to 27 of the 7C7:C5 \( V_\kappa \) DNA sequence share 100% identity with nucleotides 19157316 to 19157328 and 19624190 to 19624178 of the previously mentioned strain C57BL/6J *Mus musculus* Chromosome 12. Nucleotides 337 to 349 of the 7C7:C5 \( V_\text{H} \) DNA sequence share 100% identity with nucleotides 2502975 to 2502987 of the previously mentioned assembly of *Mus musculus* Chromosome 12. Nucleotides 350-387 of the 7C7:C5 \( V_\text{H} \) DNA sequence share 100% identity with nucleotides 25620444 to 25620407 of the previously mentioned assembly of Chromosome 12. Nucleotides 390 to 405, primarily \( C_\text{H}1^\prime \), codons of the obtained 7C7:C5 gamma 1 sequence share 100% identity with nucleotides 25521843 to 25521828. As a result, a majority of the 7C7:C5 \( V_\text{H} \) DNA sequence seems to have originated from different parts of one assembly of Chromosome 12.
In addition, much of the 7C7:C5 V<sub>H</sub> DNA sequence (including the L<sub>H</sub>) showed high similarity to predicted *Mus musculus* V<sub>H</sub> DNA sequences ref|XM_913981.1| and ref|XM_973656.2| (90% identity from nucleotide 17 to 333 of the 7C7:C5 V<sub>H</sub>) and ref|XM_001474194.1| (85% identity from nucleotide 8 to 336 of the 7C7:C5 V<sub>H</sub>). These results support that the 7C7:C5 V<sub>H</sub> is a V<sub>H</sub> that has not been previously sequenced. The amino acid sequence, however, reveals the primary structure of the antibody. The highest similarity in amino acid sequence viewed was 83% identity with gb|AAB06744.1| through amino acids 5 to 135 of the obtained 7C7:C5 gamma 1 sequence. This amino acid sequence and others were not very useful for modeling because no crystal structures are available for them.

An IgBlast was done to align known V<sub>H</sub> sequences with the 7C7:C5 V<sub>H</sub> sequence and, in turn, identify the positions of the CDRs in the V<sub>H</sub> sequence. Starting with DEQLQE, amino acids 1-30 are part of FR-H1, amino acids 31-36 are part of CDR-H1, amino acids 37-50 are part of FR-H2, amino acids 51-66 are part of CDR-H2. The remaining FR-H3, residues 67-98, CDR-H3, residues 99-105, and FR-H4, residues 106-117, were identified by a combination of Kabat numbering<sup>46</sup> and comparative analysis with other similar V<sub>H</sub> sequences since the IgBlast does not clearly indicate these regions.

**Homology Models of the 7C7:C5 Fv**

Homology models of the 7C7:C5 Fv were generated using the software program Modeller9v4. The homology modeling process required aligning the amino acid sequence of the 7C7:C5 Fv with corresponding amino acid sequences of murine IgG1 V<sub>H</sub> and V<sub>K</sub> chains (Figures 21-28). After the initial homology models were generated, the
protonation states of the models were assessed and hydrogens were added accordingly using the online software program H++. The homology models were then energy minimized by L-BFGS method. Both unbound and bound template model Fvs showed highly favorable total energies (-5582.5997 kcal/mol and -5719.1920 kcal/mol, respectively) after the minimization process. Interestingly, the bound template Fv model was energetically more favorable than the unbound template Fv model by -136.5920 kcal/mol.
## Alignments for Antigen Unbound Template Model of the 7C7:C5 Fv

<table>
<thead>
<tr>
<th></th>
<th>FR-H1</th>
<th>CDR-H1</th>
<th>FR-H2</th>
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<tbody>
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<td>D-EQLQESG--PGLVK---P-SQS-L-SLTC--S-VTGYSIT---N-NY-YW-NWIRQ-FPGNK---LEW-L-G</td>
<td></td>
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<tr>
<td>1QOK: Chain A: Residues 29-75</td>
<td>---KLQQSGAE--LVRSGT-----S-VK-LSC--TA-SGFNI-K--D-SYM---HWLRQG-P----EQGLENW-I-G</td>
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<td>1HQ4: Chain B: Residues 1-50</td>
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<tr>
<td>1S5I: Chain H: Residues 3-50</td>
<td>---QLQESG--PGLVK---P-SQS-L-SLTC--T-VTGYSIT---S-DYA-W-NWIRQ-FPGNK---LEW-M-G</td>
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<tr>
<td>2A0L: Chain D: Residues 3-50</td>
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<tr>
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</tr>
<tr>
<td>1EZV: Chain X: Residues 26-36</td>
<td>-------------------------------------GYSITSG-----Y-W-N</td>
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</tbody>
</table>

**Figure 21.** Alignment of residues 1-50 of the 7C7:C5 V\textsubscript{H} with crystal structures used for homology modeling of the antigen unbound template model of the 7C7:C5 V\textsubscript{H}. Residues in red were not aligned with the 7C7:C5 V\textsubscript{H}. Residues in black are FR residues. Residues in blue are CDR residues.
Figure 22. Alignment of residues 51-98 of the 7C7:C5 V_H with crystal structures used for homology modeling of the antigen unbound template model of the 7C7:C5 V_H. Residues in red were not aligned with the 7C7:C5 V_H. Residues in black are FR residues. Residues in blue are CDR residues.
Figure 23. Alignment of residues 99-117 of the 7C7:C5 V_H with crystal structures used for homology modeling of the antigen unbound template model of the 7C7:C5 V_H. Residues in red were not aligned with the 7C7:C5 V_H. Residues in black are FR residues. Residues in blue are CDR residues. Residues 118-122 in plum are the first five residues of the C_H1, which were also aligned for generating the model.
Figure 24. Alignment of residues (a) 1-68 and (b) 69-105 of the 7C7:C5 $\kappa$ with crystal structures used for homology modeling of the antigen unbound template model of the 7C7:C5 $\kappa$. Residues in red were not aligned with the 7C7:C5 $\kappa$. Residues in black are FR residues. Residues in blue are CDR residues. Residues 106-111 in plum are the first six residues of the $\kappa$, which were also aligned for generating the model.

### (a)

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<tr>
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<th>FR-L3</th>
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<tr>
<td>1QOK: Chain A: Residues 162-229</td>
<td>EN-VLTQSP-AIM-SASP-GEKVTITCSASSSVSYHMWWFQQKPF-GTSPKL--WYST---SN-LASGVPARFSGSGST</td>
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<td>1E6O: Chain L: Residues 1-68</td>
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<td>2FAT: Chain L: Residues 1-68</td>
<td>D-IVLTQSPD-I-TAAS-LGQKVTITCSASSSVSYHMWWYQQK-SGTSPK--EI--KLasGVPARFSGSGST</td>
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### (b)

<table>
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<th>FR-L4</th>
<th>$\kappa$</th>
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<td>SYSLTISR-SMEAEDAAT-IYYCQQ-R--SYPLTFGA-GGTKLEIKRA</td>
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<td></td>
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<td>1E6O: Chain L: Residues 69-111</td>
<td>SYSLTIS-SMEAEDAA-IYYCQQ-W--NYP-PFTGA-GGTKLEIKRA-DAAP</td>
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<tr>
<td>2FAT: Chain L: Residues 69-111</td>
<td>SYSLTIS-SMEAEDAA-IYYCQQ-W--NYP-PFTGA-GGTKLEIKRA-DAAP</td>
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<tr>
<td>1AIF: Chain L: Residues 95-114</td>
<td>-----------------------------YP-YTFA-GGTKLEIKRA-DAAP</td>
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Alignments for Antigen Bound Template Model of the 7C7:C5 Fv

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<thead>
<tr>
<th>FR-H1</th>
<th>CDR-H1</th>
<th>FR-H2</th>
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<tr>
<td>7C7:C5: Chain H: Residues 1-50</td>
<td>D-EQLQESG--PGLVK---P-SQS--L-SLTC--T-VTGYSIT---N-NY--YW--NWIRQ--FPGNK---LEW-M-G</td>
<td></td>
</tr>
<tr>
<td>1QOK: Chain A: Residues 29-75</td>
<td>---KLQSGAE--LVRSGT----SVK--LSC--TA-SGFINI-K--D-SYM---HWLRQG-P----EQGLEN-I-G</td>
<td></td>
</tr>
<tr>
<td>1ORS: Chain B: Residues 1-50</td>
<td>DV--QLQSGE--PGLVK---P-SQS--L-SLTC--T-VTGYSIT---N-NYA--W--NWIRQ--FPGNK---LEW-M-G</td>
<td></td>
</tr>
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<td>1CF8: Chain H: Residues 1-50</td>
<td>DV--QLQSGE--PGLVK---P-SQS--L-SLTC--T-VTGYSIT---SG--YA--W--NWIRQ--FPGNK---LEW-M-G</td>
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<td>1BAF: Chain H: Residues 1-50</td>
<td>DV--QLQSGE--PGLVK---P-SQS--L-SLTC--T-VTGYSIT---S-DYA--W--NWIRQ--FPGNK---LEW-M-G</td>
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<tr>
<td>1KCR: Chain H: Residues 4-50</td>
<td>---LQESG--PGLVK---P-SQS--L-SLTC--T-VTGYSIT---S-DYA--W--NWIRQ--FPGNK---LEW-M-G</td>
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<tr>
<td>1KEN: Chain H: Residues 4-50</td>
<td>---LQESG--PGLVK---P-SQS--L-SLTC--T-VTGYSIT---S-DYA--W--NWIRQ--FPGNK---LEW-M-G</td>
<td></td>
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<tr>
<td>2AOL: Chain D: Residues 3-50</td>
<td>---QLSG--PGLVK---P-SQS--L-SLTC--T-VTGYSIT---N-NYA--W--NWIRQ--FPGNK---LEW-M-G</td>
<td></td>
</tr>
<tr>
<td>1BGX: Chain H: Residues 5-50</td>
<td>-----QESG--PGLVK---P-SQS--L-SLSC--T-VTGYSIT---S-DYA--W--NWIRQ--FPGNK---LEW-M-G</td>
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<td>1KC5: Chain H: Residues 3-50</td>
<td>---KLQSGE--PGLVK---P-SQS--L-SLTC--T-VTGYSIT---S-DYA--W--NWIRQ--FPGNK---LEW-M-G</td>
<td></td>
</tr>
<tr>
<td>1EZV: Chain X: Residues 26-36</td>
<td>------------------------------GYSISITSG----Y-YW-N</td>
<td></td>
</tr>
<tr>
<td>1SS5I: Chain H: Residues 3-48</td>
<td>---QLSG--PGLVK---P-SQS--L-SLTC--T-VTGYSIT---S-DYA--W--NWIRQ--FPGNK---LEW</td>
<td></td>
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</tbody>
</table>

Figure 25. Alignment of residues 1-50 of the 7C7:C5 V_H with crystal structures used for homology modeling of the antigen bound template model of the 7C7:C5 V_H. Residues in red were not aligned with the 7C7:C5 V_H. Residues in black are FR residues. Residues in blue are CDR residues.
Figure 26. Alignment of residues 51-98 of the 7C7:C5 V_H with crystal structures used for homology modeling of the antigen bound template model of the 7C7:C5 V_H.

Residues in red were not aligned with the 7C7:C5 V_H. Residues in black are FR residues. Residues in blue are CDR residues.
<table>
<thead>
<tr>
<th>CDR-H3</th>
<th>FR-H4</th>
<th>C_H1</th>
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<tbody>
<tr>
<td>7C7:C5: Chain H: Residues 99-122</td>
<td>Y--GW----LP----L-AWYG-QGT-LVTVS-A--KTTP</td>
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</tr>
<tr>
<td>1QOK: Chain A: Residues 127-146</td>
<td>-PTG--PYYP--D--------WYG-QGT-VTVS-S------</td>
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<tr>
<td>1ORS: Chain H: Residues 100-123</td>
<td>YD--YFAMD---------WYG-QGTS-VTVS-S--KTTP</td>
<td></td>
</tr>
<tr>
<td>1CF8: Chain H: Residues 99-121</td>
<td>---GYGNSD---------WYG-QGT-LVTVS-A--KTTP</td>
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<tr>
<td>1F90: Chain H: Residues 99-123</td>
<td>YDD--YTWFT---------WYG-QGT-LVTVS-A--KTTP</td>
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<td>1BAF: Chain H: Residues 99-120</td>
<td>---GW-----P---L-AWYG-QGTQ-VSVSE-A--KTTP</td>
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<tr>
<td>1KCR: Chain H: Residues 99-121</td>
<td>---G--GTG--FT-----WGA--GT-LVTVSAAA--TTTP</td>
<td></td>
</tr>
<tr>
<td>1KEN: Chain H: Residues 99-125</td>
<td>P--Y--DYD-----FFF--D--WYG-QGTT-LTVS-S--KTTP</td>
<td></td>
</tr>
<tr>
<td>2A0L: Chain H: Residues 100-116</td>
<td>Y--D--YFA--------MD--WYG-QGTS-VTV</td>
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<tr>
<td>1BGX: Chain H: Residues 101-123</td>
<td>Y--GYWYFD--V------WYG-QGTT-LTVS-S--KTTP</td>
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</tr>
<tr>
<td>1KC5: Chain H: Residues 99-121</td>
<td>---G--GTGF--D-----WGA--GTT-LVTVS-AAA--TTTP</td>
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<td>1NCW: Chain H: Residues 101-123</td>
<td>-----WYDGG--AGS-----WYG-QGT-LVTVS-A--KTTP</td>
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<td>3CFD: Chain H: Residues 101-125</td>
<td>Y--G--NYGR--GD-----WYG-QGTS-VTVS-S--KTTP</td>
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</tr>
<tr>
<td>1KCS: Chain H: Residue 100</td>
<td>-----G</td>
<td></td>
</tr>
<tr>
<td>2FON: Chain A: Residues 122-127</td>
<td>-----W-----LP-----L--AY</td>
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</tr>
<tr>
<td>191I: Chain H: Residues: 99-123</td>
<td>Y--GYGYG---------L-AWYG-QGT-LVTVS-A--KTTP</td>
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</table>

Figure 27. Alignment of residues 99-117 of the 7C7:C5 V_H with crystal structures used for homology modeling of the antigen bound template model of the 7C7:C5 V_H. Residues in red were not aligned with the 7C7:C5 V_H. Residues in black are FR residues. Residues in blue are CDR residues. Residues 118-122 in plum are the first five residues of the C_H1, which were also aligned for generating the model.
<table>
<thead>
<tr>
<th>FR-L1</th>
<th>CDR-L1</th>
<th>FR-L2</th>
<th>CDR-L2</th>
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<table>
<thead>
<tr>
<th>FR-L3</th>
<th>CDR-L3</th>
<th>FR-L4</th>
<th>Cκ</th>
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</table>

Figure 28. Alignment of residues (a) 1-68 and (b) 69-105 of the 7C7:C5 Vκ with crystal structures used for homology modeling of the antigen bound template model of the 7C7:C5 Vκ. Residues in red were not aligned with the 7C7:C5 Vκ. Residues in black are FR residues. Residues in blue are CDR residues. Residues 106-111 in plum are the first six residues of the Cκ, which were also aligned for generating the model.

To compare the overall structure of the 7C7:C5 Fv models with its crystal structure templates, the template structures were superimposed on three different reference structures (the 7C7:C5 Fv models; the complete Fv template, 1QOK; and the most similar template in primary structure to the 7C7:C5 VH or Vκ) using DeepView/Swiss-PDB Viewer. The Cα RMSDs for each superimposed structure were calculated also in DeepView/Swiss-PDB Viewer. Tables 5 and 6 show Cα RMSDs of the
areas aligned for generation of the 7C7:C5 Fv models. Overall, the C\textsubscript{\alpha} RMSD is consistent among templates. The most similar template in primary structure to the 7C7:C5 Fv shows the smallest C\textsubscript{\alpha} RMSD when superimposed with all the other templates. The 1QOK template shows an overall two fold larger C\textsubscript{\alpha} RMSD than the most similar template in primary structure to the 7C7:C5 Fv, when superimposed with all the other templates.
Table 5. The Cα RMSD for each crystal structure template aligned in generation of the antigen unbound model of the 7C7:C5 Fv

VH Unbound Template Model Cα RMSD

<table>
<thead>
<tr>
<th>Structure</th>
<th>RMSD (Å)</th>
<th>RMSD (Å)</th>
</tr>
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<tr>
<td>1QOK</td>
<td>1.58</td>
<td>reference</td>
</tr>
<tr>
<td>1HQ4</td>
<td>1.35</td>
<td>1.06</td>
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<td>1F8T</td>
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<td>1S5I</td>
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<td>1.10</td>
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<td>32C2</td>
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<tr>
<td>2FON</td>
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<td>WLP 0.25, LAY 0.66</td>
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<tr>
<td>1I9I</td>
<td>1.73</td>
<td>excluding YGY 1.20</td>
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VK Unbound Template Model Cα RMSD

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<th>RMSD (Å)</th>
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*7C5U is the energy minimized antigen unbound model of the 7C7:C5 Fv. Reference indicates structure in PDB format upon which other structures were superimposed. RMSDs highlighted in blue are for the indicated parts of the template. NA = not applicable
Table 6. The $C_\alpha$ RMSD for each crystal structure template aligned in generation of the antigen bound model of the 7C7:C5 Fv

**V$_H$ Bound Template Model $C_\alpha$ RMSD**

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</tr>
<tr>
<td>1S51</td>
<td>1.30</td>
</tr>
<tr>
<td>2FON</td>
<td>3.51</td>
</tr>
<tr>
<td>1I9I</td>
<td>2.20</td>
</tr>
</tbody>
</table>

*7C5V is the energy minimized antigen bound model of the 7C7:C5 Fv. Reference indicates structure in PDB format upon which other structures were superimposed. RMSDs highlighted in blue are for the indicated parts of the template. NA = not applicable

**V$_\kappa$ Bound Template Model $C_\alpha$ RMSD**

<table>
<thead>
<tr>
<th>Structure</th>
<th>RMSD (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7C5V</td>
<td>reference</td>
</tr>
<tr>
<td>1QOK</td>
<td>1.06</td>
</tr>
<tr>
<td>1E6J</td>
<td>1.06</td>
</tr>
<tr>
<td>2FD6</td>
<td>1.24</td>
</tr>
<tr>
<td>2FBJ</td>
<td>1.28</td>
</tr>
</tbody>
</table>

The $C_\alpha$ RMSDs with the 7C7:C5 Fv as the reference structure are the largest $C_\alpha$ RMSDs among the three reference structures. Nevertheless, this result is quite logical for
a few reasons. First, the Cα deviations in the 7C7:C5 Fv models are a combination of the deviations of 1QOK and the other templates used specifically for the VH or VK. Thus, the Cα deviation of the templates superimposed on the 7C7:C5 Fv models is greater than that of the other templates when superimposed on 1QOK, but is not necessarily additive as much overlap in deviation occurs among templates keeping the overall Cα RMSDs of the 7C7:C5 Fv models reasonable.

Second, 1QOK was first the template listed in the alignment file and, thus, it is expected to have set the initial framework for generating the 7C7:C5 Fv models. It has the lowest amino acid sequence identity of the templates used for generating either the 7C7:C5 VH or VK and was selected only because the other templates showed high identity in either the VH or VK but not both. The template 1QOK had the highest amino acid sequence identity of a complete Fv with the 7C7:C5 Fv. As a result, the VH and VK of the 7C7:C5 Fv are a relatively unique combination that are expected to vary in association from its templates, especially after the energy minimization process.

Lastly, the CDR-H3 had a rather limited selection of templates to homology model it. As a result, only the Cα RMSDs calculated with the 7C7:C5 Fv models as reference points included any residues of the CDR-H3 that aligned with a superimposed template structure. The two template reference structures in Tables 5 and 6 excluded residues of the CDR-H3 due to the lack of similar residues in this area in a majority of the templates. The templates 2FON and 1I9I both show relatively higher Cα RMSDs than the other templates when superimposed on the 7C7:C5 Fv models. The residues of the CDR-H3 showed much higher Cα RMSDs than any of the other CDRs.
Table 7 shows $C_\alpha$ RMSDs calculated for CDR-H3 and L2 (the CDRs that comprise the majority of the predicted binding pocket). The template 1BAF appears to have resulted in the lowest $C_\alpha$ RMSD for the CDR-H3 and aligns with six of the eight residues in this CDR. The templates 1I9I and 2FON also align with six of the eight residues in this CDR. The overall 1I9I template has a reasonable $C_\alpha$ RMSD when superimposed with the 7C7:C5 CDR-H3, particularly if the residues 99-Y, 101-GY-102 of the 1I9I template and 99-YGW-101 of the 7C7:C5 $\text{V_H}$ are excluded from the alignment. Also, if the alignment of 2FON with the 7C7:C5 CDR-H3 is broken into two parts (WLP and LAY), then the $C_\alpha$ deviation is barely noticeable. The major $C_\alpha$ deviation appears to originate from the Pro H103 to Leu H104 linkage. The conformation of this linkage appears to be more consistent with the 1BAF template, as suggested by the lower $C_\alpha$ deviations from this template in the CDR-H3 area.
Table 7. The Cα RMSDs for CDRs of the predicted binding pocket of the 7C7:C5 Fv

CDR-H3 Antigen Bound Templates

<table>
<thead>
<tr>
<th>Structure</th>
<th>RMSD (Å)</th>
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<th></th>
</tr>
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<tbody>
<tr>
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<td></td>
</tr>
<tr>
<td>1BAF</td>
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<td>2.44</td>
<td></td>
</tr>
<tr>
<td>1I9I</td>
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CDR-L2 Antigen Bound Templates

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<th></th>
</tr>
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<tbody>
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</tr>
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<td></td>
<td>0.19</td>
<td></td>
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</tbody>
</table>

*7C5V is the energy minimized antigen bound model of the 7C7:C5 Fv. Reference indicates structure in PDB format upon which other structures were superimposed. RMSDs highlighted in blue are for the indicated parts of the template.

Models were assessed for stereochemical quality using the software program PROCHECK before and after an energy minimization. Results of the PROCHECK assessment before minimization actually showed high percentages of residues (88.6% of residues for the unbound-template model and 89.1% of residues for the bound-template model) in the most favored backbone conformations; however, total energy calculations for both of these models were highly unfavorable, particularly due to overextended bond distances between residues. PROCHECK assessment was performed for the bound template model after minimization by L-BFGS method. The unbound template model underwent the same minimization process as the bound template model and, thus, assessment of the stereochemical quality was only deemed necessary for the more important of the two models.
A Ramachandran plot for determining the stereochemical quality of the bound template model of the 7C7:C5 Fv is shown in Figure 29. The plot shows that 147 residues (72.8% of residues) are in the most favored conformations; 45 residues (22.3% of residues) are in the additional allowed conformations; 8 residues (4.0% of residues) are in the generously allowed conformations, and 2 residues (1.0% of residues) are in disallowed conformations. Based on this plot, 99% of all residues in the minimized bound template model of the 7C7:C5 Fv were found to be in allowed conformations.

Figure 29. Ramachandran plot generated by PROCHECK for the L-BFGS minimized bound template model of the 7C7:C5 Fv using an applied cutoff resolution of 2.0 Å.
Another significant assessment showed that the minimized model contained no bad contacts, or contacts in which one residue is intruding into another residue’s spatial arrangement. Also, no main-chain bond distances varied from “ideal” bond distances by more than 0.05Å. Lastly, only three main-chain bond angles (Thr H114-Val H115, Pro H122, and Pro H122-Glu L1) in the entire model were slightly greater than 10° of “ideal” bond angles and all three of these bond angles were at or near the amino terminus of the C\text{H}1 domain and, thus, none of the residues involved in these bond angles were deemed likely to have a significant effect on antigen binding as well as overall model quality.

Two separate models of the 7C7:C5 Fv were generated. One of the models, referred to as the unbound template model, was generated primarily using V\text{H} and V\text{\kappa} chain coordinate data from crystallographic structures of Fabs in conformations with no antigen bound. The other model, referred to as the bound template model, was generated primarily using V\text{H} and V\text{\kappa} chain coordinate data from crystallographic structures of Fabs in antigen bound conformations as templates. Only the bound template model was used for docking studies due to the likelihood that crystallographic structures of Fabs in an antigen bound form should give a better prediction of a suitable binding pocket. The reasoning behind this assumption is that the Fvs of Fabs undergo induced fit conformations that influence binding activity and binding pocket formation\textsuperscript{82,123-125}. In fact, in some cases a binding pocket is not even noticeable until after the appropriate conformational changes in the Fv occur during the antigen binding process\textsuperscript{82}.

Although only a theoretical prediction, visual comparison of the predicted binding pocket of the 7C7:C5 Fv in the energy minimized unbound and bound template models shown in Figure 30 demonstrates a good example of the possible differences between the
conformation of the binding pocket area of an unbound and an antigen-bound Fv. In the unbound template Fv model, no obvious binding pocket is noticeable in the highlighted region. The bound template Fv model, on the other hand, shows a binding pocket that is not only obvious in appearance, but appears to be a custom fit for a single d^{6m}A antigen based on docking studies discussed next in this paper.

Figure 30. Comparison of the two energy minimized models of the 7C7:C5 Fv. Two different viewpoints of (a) the (left top and bottom) model generated primarily using crystallographic V_{H} and V_{κ} structures of Fabs in antigen unbound conformations as templates and (b) the (right top and bottom) model generated primarily using crystallographic V_{H} and V_{κ} structures of Fabs in antigen bound conformations as templates. Comparison of the highlighted (dotted) region between the model images on the left and the corresponding model images on the right clearly shows the presence of a binding pocket in the model on the right that is not present in the model on the left. The highlighted residues included His L33, Tyr L35, Pro L45, Ile L47, Tyr L48, Ser L55, Leu H102, Pro H103, Leu H104, and Ala H105 and were based on paratope residues found in docking studies with d^{6m}A.

**Color Scheme**

- White = V_{H} FR
- Yellow = V_{κ} FR
- Cyan = CDR-H1
- Blue = CDR-H2
- Green = CDR-H3
- Red = CDR-L1
- Pink = CDR-L2
- Purple = CDR-L3
Rigid Fv and Flexible Ligand Docking Analysis of the 7C7:C5 Fv

Preliminary “blind docking” studies using wide-range grid boxes covering much of the top (viewpoint away from the amino termini of the constant regions and the carboxyl termini of the variable regions) center interface between the V_H and V_κ chains (including areas proximal to the heavy and light CDR interface) were done to help predict the binding pocket. The preliminary docking studies were all performed using rigid body docking for the Fv and flexible docking for the antigen. Docking studies were performed with the grid box centered at different positions along the upper V_H and V_κ interface. The docking experiment that revealed a docked conformation for d^6mA with the most favorable (most negative) binding energy was selected as the most likely area for the paratope.

The most favorable binding energy found for d^6mA was -5.15 kcal*mol^{-1}*K^{-1} and the corresponding dissociation constant, K_d, (inverse of the association constant, K_a) was 1.69 x 10^{-4} M. The selected paratope area visually appears to be a binding pocket that seems to fit a single d^6mA securely (Figure 31a, b). Furthermore, rigid body docking of dA and ssDNA trimer (A^6mA-T) each revealed at least one conformation which highly resembled the most favorable conformation for d^6mA binding (Figure 31c-f). In fact, the most favorable rigid body docking of d^6mA and dA (Figure 31a-d) seem to be virtually identical conformations, in which the main difference is that d^6mA has a methyl group instead of a hydrogen in the N6 position of the adenine base sticking down towards the deepest portions of the binding pocket. The docking energies for dA and A^6mA-T were -3.56 kcal*mol^{-1}*K^{-1} and -2.37 kcal*mol^{-1}*K^{-1}, respectively. The corresponding K_d for dA and A^6mA-T were 2.46 x 10^{-3} M and 1.82 x 10^{-2} M, respectively.
Figure 31. Preliminary “blind docking” studies using a wide range grid box, a rigid body model of the 7C7:C5 Fv, and a flexible antigen. The top (a) (b) models show binding of the 7C7:C5 Fv to d$_{6m}$A. The middle (c) (d) models show binding of the 7C7:C5 Fv to dA. The bottom (e) (f) models show binding of the 7C7:C5 Fv to a ssDNA trinucleotide of deoxyadenosine-deoxy-N$^6$-methyladenosine-deoxythymidine (A-$^{6m}$A-T). Atomic color code (grey for carbon and non-polar hydrogens, cyan for polar hydrogens, blue for nitrogen, and red for oxygen) was used for docked antigens. See color code in Figure 30 for the Fv, p.100.
The docking interactions of the models with rigid binding pocket residues were analyzed using ADT-1.5.2. van der Waals (vdW) interactions were determined using a scaling factor of 1.3 to adjust the default vdW distance (the sum of the two radii of the interacting atoms) for the purpose of allowing complete interaction of the adenine base and the methyl group in the N6 position. Adjustment of this scaling factor should help compensate for contacts that may be off simply due to the rigidity of the model Fv. The predicted interactions between the paratope of the anti-d\textsuperscript{6m}A Fv and the epitopes of d\textsuperscript{6m}A, dA, and A\textsuperscript{-6m}A-T are shown in Figure 32, p. 106.

The paratope for d\textsuperscript{6m}A binding includes the following eleven residues: His L33 (=His 155), Tyr L35 (=Tyr 157), Pro L45 (=Pro 167), Ile L47 (=Ile 169), Tyr L48 (=Tyr 170), Ala L54 (=Ala 176), Ser L55 (=Ser 177), Leu H102 (=Leu 102), Pro H103 (=Pro 103), Leu H104 (=Leu 104), and Ala H105 (=Ala 105). The residues Leu H102 and Ser L55 show hydrogen (H) bonds to the d\textsuperscript{6m}A molecule, in which the main-chain carbonyl group of Leu H102 receives the lone hydrogen in the N6 position of adenine and the main chain NH group of Ser L55 donates a hydrogen to the N3 of adenine. A total of five polar contacts are made with d\textsuperscript{6m}A, of which approximately two interactions are with the deoxyribose moiety, two interactions are made with nitrogen atoms of the adenine ring system, and one interaction is made with the N6 lone hydrogen. The sum of the contacts made with the epitope, especially for dispersion interactions, are not necessarily equal to the total number of contacts made against each specific component since some degree of overlap for contacts occurs between regions. A total of thirty-two dispersion contacts are made with d\textsuperscript{6m}A, of which approximately two to four show interaction with the
deoxyribose moiety, twenty-one show interaction with the adenine base, and thirteen show interaction with the N6 methyl group.

The paratope for dA binding includes the following eight residues: Pro L45, Tyr L48, Ala L54, Ser L55, Leu H102, Pro H103, Leu H104, and Ala H105. The residues Leu H102 and Ser L55 show H-bonds to the dA molecule, in which the main-chain carbonyl oxygen of Leu H102 receives the N6 hydrogen sticking up and away from the bottom of the binding pocket and the main chain NH group of Ser L55 donates a hydrogen to the N3 of adenine. A total of six polar contacts are made with dA, of which approximately two are made with the deoxyribose moiety, one is with a nitrogen of the adenine ring system, and three are with the N6 and the N6 hydrogens. A total of twenty-two hydrophobic contacts are made with dA, of which four show interaction with the deoxyribose moiety, eighteen show interaction with the adenine base, and four show interaction with the N6 hydrogens.

An extended range of the paratope occurs to accommodate the larger volume of the ssDNA trinucleotide (A-^{6m}A-T). The central or primary paratope in this case includes any interactions that involve binding of the d^{6m}A moiety, which includes the following six residues: Tyr L48, Ala L54, Ser L55, Leu H102, Pro H103, and Ala H105. No H-bonds occurred with the d^{6m}A moiety, but interestingly a hydrophobic π-stacking interaction occurred between Tyr L48 and the adenine ring on the side of d^{6m}A facing the dT nucleotide. A total of one polar contact is made with the deoxyribose of the d^{6m}A moiety. A total of sixteen dispersion contacts are made with the d^{6m}A moiety, of which two show interaction with the deoxyribose moiety, ten show interaction with the adenine
ring system, and eleven show interaction with the N6 substituents (approximately eight contacts to the methyl group and three contacts to the lone hydrogen).

Secondary and tertiary paratopes are present for the nucleotides neighboring the \( d^{6m}A \) moiety. The residues Tyr L48, Glu L49 (\( \approx \)Glu 171), Lys L52 (\( \approx \)Lys 174), and Tyr H33 (\( \approx \)Tyr 33) contribute a total of six polar contacts to the A-\( 6m^{e}A \) and \( 6m^{a}A-T \) phosphate groups, respectively. A total of four dispersion contacts are made with the two phosphate groups by Glu L49, Tyr H33, and Trp H101 (\( \approx \)Trp 101). Only two residues interact with the dA moiety: Asn H32 (\( \approx \)Asn 32) and Tyr H33. The carboxamide oxygen of Asn H32 receives a hydrogen from one of the N6 hydrogens of adenine. Six dispersion and three polar contacts are made with the dA moiety. Three residues interact with the dT moiety: Tyr L48, Glu L49, and Lys L52. The oxygen of the phenolic hydroxyl group of Tyr L48 receives a hydrogen from the alcohol group of the deoxyribose moiety. Four dispersion and two polar contacts are made with the dT moiety.
Figure 32. Predicted paratope interactions of the anti-\(d^{6m}A\) Fv using a rigid binding pocket with flexible (a) (b) deoxy-\(N^6\)-methyladenosine, (c) (d) unmethylated deoxyadenosine, and (e) (f) ssDNA trinucleotide (A-\(6m^6A\)-T). Black dotted lines indicate H-bonds. Grey spheres indicate hydrophobic interactions and smaller light blue or red spheres indicate the presence of a polar group involved in either a dispersion or a dipole interaction. The maximal distance from the epitope for a van der Waals contact was the sum of the two radii of the interacting atoms with a scaling factor of 1.3.
The paratopes of the rigid 7C7:C5 Fv binding pocket for each of the three docked antigens (d\textsuperscript{6m}A, dA, and A-\textsuperscript{6m}A-T) include the following common residues: Tyr L48, Ala L54, Ser L55, Leu H102, Pro H103, and Ala H105. Apparently, Tyr L48, Ser L55, and Pro H103 are the most critical binding residues based on the rigid body Fv docking models with flexible antigens. They comprise a large number of the overall contacts, in which Tyr L48 has the most contacts generally followed by Pro H103 and lastly Ser L55. The docking of d\textsuperscript{6m}A receives more paratope residues than either dA or d\textsuperscript{6m}A moiety of A-\textsuperscript{6m}A-T. While docking of A-\textsuperscript{6m}A-T provides interaction with twelve paratope residues total versus eleven paratope residues for d\textsuperscript{6m}A and eight paratope residues for dA, the docking of A-\textsuperscript{6m}A-T occurs over a much wider volume. Thus, the presence of more paratope or paratope interactions alone does not necessarily mean a stronger binding affinity since K\textsubscript{d} is relative to the number of interactions and type of interactions per volume of encountered epitope.

In the docking of d\textsuperscript{6m}A, residues His L33, Tyr L35, Pro L45, and Leu H104 show interactions with the N6 methyl group. The overall number of interactions with the N6 methyl group of d\textsuperscript{6m}A alone and d\textsuperscript{6m}A in A-\textsuperscript{6m}A-T is thirteen and eight, respectively. The overall number of interactions with the N6 hydrogen of dA that sticks downwards into the binding pocket is two, of which one is a polar interaction and the other is a dispersion interaction. Table 8 summarizes the overall number of interactions by category: stacking, H-bond, polar, or dispersion interactions.
Table 8. Summary of the total interactions of the rigid 7C7:C5 Fv with the flexible antigens: d^{6m}A, dA, and ssDNA (A^{-6m}A-T)

<table>
<thead>
<tr>
<th>Type of Interactions</th>
<th>d^{6m}A</th>
<th>dA</th>
<th>ssDNA (A^{-6m}A-T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>π-Stacking Interactions</td>
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<td>1</td>
</tr>
<tr>
<td>Hydrogen Bonds</td>
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<td>2</td>
</tr>
<tr>
<td>Polar Contacts</td>
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<td>12</td>
</tr>
<tr>
<td>Dispersion Contacts</td>
<td>32</td>
<td>22</td>
<td>30</td>
</tr>
</tbody>
</table>

7C7:C5 Fv with Five Flexible Binding Pocket Residues and Flexible Antigen Docking

The calculated K_d values from the rigid Fv model docking studies suggest relatively weak binding for all three antigens (d^{6m}A, dA, and A^{-6m}A-T); however, antigen binding pockets are not rigid. By allowing for some of the residues around the binding pocket to be flexible, more contacts as well as tighter contacts should occur between paratope and epitope. Accordingly, the affinity for d^{6m}A, dA, and A^{-6m}A-T should improve as a result of the ability of paratope residues to form more of a custom fit for each epitope. To test this hypothesis, docking studies were performed with a model of the anti-d^{6m}A Fv that included flexible residues in an area around the binding pocket. Five residues (Tyr L35, Ile L47, Tyr L48, Ser L55, and Leu H104) around the predicted binding pocket were selected to be flexible.

The flexible residues added a total of twelve active torsions to the overall amount of active torsions. The only other torsions allowed were the same active ligand torsions used in the rigid body docking studies. In addition to adding flexible residues to the study, a few search parameters related to energy evaluations and the overall
conformational output were increased and a more focused grid box was applied. A collection of the flexible residue docking results is shown in Figure 33, p. 112. Antigen binding for d\(^6\)m\(^A\) and d\(A\) is limited to CDR-H3 and –L2. In fact, the predicted binding pocket lies at the interface between CDR-H3 and –L2 and includes a couple of framework residues according to Kabat numbering scheme for CDRs. Conformations for d\(A\) and ssDNA trimer (A–6m\(^A\)-T) were selected based on proximity to the most favorable conformation for d\(^6\)m\(^A\) since d\(^6\)m\(^A\) is the closest in structure of these three antigens to 6m\(^A\)os, the antigen to which the 7C7:C5 MAb was produced.

The most favorable conformation for d\(^6\)m\(^A\) binding to the 7C7:C5 binding pocket is shown in Figure 33a, b. The binding energy for this conformation of d\(^6\)m\(^A\) was -14.57 kcal\*mol\(^{-1}\)\*K\(^{-1}\) and the corresponding K\(_d\) was 2.10 \times 10^{-11} M. The docking of d\(^6\)m\(^A\) in the binding pocket with flexible residues shows similarity to the binding of d\(^6\)m\(^A\) in the rigid body binding pocket, in which the binding of d\(^6\)m\(^A\) is once again deep within the binding pocket and the N6 methyl group is sticking down towards the deepest portions of the binding pocket. The selected conformation for d\(A\) binding (shown in Figure 33c, d) was not the most favorable conformation for d\(A\) binding to the 7C7:C5 binding pocket in terms of binding energy; however, it was the closest in appearance to the most favorable conformation for d\(^6\)m\(^A\) binding.

Besides, the most favorable energy conformations for d\(A\) found in the study involved the deoxyribose moiety fitting more deeply into the binding pocket in which the adenine base was sticking out of the binding pocket with minimal Fv interaction. None of these more favorable end conformations for d\(A\) seemed reasonable on that basis. Also, none of them would be applicable to d\(A\) in ssDNA since the connection of the sugar
moiety to phosphates and other nucleotides would prevent the sugar moiety from binding deep into the binding pocket. The binding energy for the selected dA conformation is -13.45 kcal mol\(^{-1}\)K\(^{-1}\) and the corresponding \(K_d\) is \(1.39 \times 10^{-10}\) M. The conformation for dA does not bind as deep into the binding pocket as \(d^{6m}A\). In fact, the N6 hydrogens of dA appear to interact with residues closer to the surface. In turn, the adenine base of dA fits into the binding pocket at more of a diagonal orientation than the adenine base of \(d^{6m}A\), which fits into the pocket in a nearly vertical orientation. The diagonal orientation of dA results in the deoxyribose moiety being closer to Ser L55 rather than Tyr L48.

The docked conformation of the \(d^{6m}A\) moiety of the ssDNA trinucleotide (A-\(d^{6m}A-T\)) to the 7C7:C5 Fv (in Figure 33e, f) resembled the most favorable conformation for the binding of \(d^{6m}A\) alone to the 7C7:C5 Fv. As a result, this binding mode was selected as the most probable binding conformation for A-\(d^{6m}A-T\) found. The binding energy for this selected conformation of A-\(d^{6m}A-T\) was -14.36 kcal/mol and the corresponding \(K_d\) is 2.963 \(\times 10^{-11}\) M. Coincidentally, the selected conformation of A-\(d^{6m}A-T\) (shown in Figure 33e, f) also happened to be the most favorable conformation for A-\(d^{6m}A-T\) binding found. All three nucleotides of A-\(d^{6m}A-T\) appear to make contacts with the surface of the Fv extending the paratope over a larger surface area than with either \(d^{6m}A\) or dA. Similar to the conformation found for dA docking in Figure 33c, d, the selected conformation of A-\(d^{6m}A-T\) docking appears to bind the \(d^{6m}A\) moiety not as deep into the binding pocket as \(d^{6m}A\) alone.

The \(d^{6m}A\) moiety of the trinucleotide A-\(d^{6m}A-T\) may not be able to bind as deep as \(d^{6m}A\) merely because it is connected to two other nucleotides that sterically prevent it from binding deeper into the binding pocket. In addition, interactions with the phosphate
groups between nucleotides as well as interactions with each of the nucleotides may also prevent the $d^{6m}A$ moiety in A-$6mA$-T from binding as deep into the binding pocket as $d^{6m}A$ alone. The possibility of a secondary binding pocket that opens up and forms after induced fit binding of a single $d^{6m}A$ in the primary binding pocket exists, but such information would need to be determined experimentally first.
Figure 33. Docking studies using a more focused grid box than in preliminary docking studies, a set of five flexible residues (Tyr L35, Ile L47, Tyr L48, Ser L55, and Leu H104) in the area of the binding pocket of the model 7C7:C5 Fv, and a flexible antigen. The top (a) (b) models show binding of the 7C7:C5 Fv to d6mA. The middle (c) (d) models show binding of the 7C7:C5 Fv to dA. The bottom (e) (f) models show binding of the 7C7:C5 Fv to A-6mA-T. Atomic color code (grey for carbon and non-polar hydrogens, cyan for polar hydrogens, blue for nitrogen, and red for oxygen) was used for docked antigens. See color code in Figure 30 for the Fv, p.100.
The docking interactions of the models with flexible Fv residues were analyzed using ADT-1.5.2. Van der Waals interactions were determined using a scaling factor of 1.3 to adjust the default vdW distance for the purpose of allowing complete interaction of the adenine base and the methyl group in the N6 position. Plus, adjustment of this scaling factor should help compensate for contacts that may be off simply due to the overall general rigidity of the model Fv. The predicted interactions between the paratope of the anti-d^6mA Fv and the epitopes of d^6mA, dA, and A-^6mA-T are shown in Figure 34, p. 116. The paratope for d^6mA binding includes the following eleven residues: His L33, Tyr L35, Pro L45, Ile L47, Tyr L48, Ala L54, Ser L55, Leu H102, Pro H103, Leu H104, and Ala H105.

The residues Leu H102, Tyr L48, and Ser L55 show hydrogen bonds to the d^6mA molecule, in which the main-chain carbonyl group of Leu H102 receives the lone hydrogen in the N6 position of adenine, the phenolic hydroxyl group of Tyr L48 donates a hydrogen to the 5’ oxygen of the deoxyribose moiety, and the main chain NH group of Ser L55 donates a hydrogen to the N3 of adenine. A total of six polar contacts are made with d^6mA, of which approximately two interactions are with the deoxyribose moiety, two interactions are with the N3 of adenine, one interaction is with the N5 of adenine, and one interaction is with the N6 lone hydrogen. A total of twenty-eight dispersion contacts are made with d^6mA, of which approximately five show interaction with the deoxyribose moiety, sixteen show interaction with the adenine base, and thirteen show interaction with the N6 methyl group.

The paratope for dA binding includes the following seven residues: Tyr L48, Glu L49, Lys L52, Ser L55, Leu H102, Pro H103, and Leu H104. The residues Glu L49 and
Ser L55 show H-bonds to the dA molecule, in which the carbonyl oxygen of the side-chain of Glu L49 receives the N6 hydrogen sticking down into the pocket and the oxygen of the side-chain hydroxyl group of Ser L55 receives a hydrogen from the alcohol group of the deoxyribose moiety. A total of six polar contacts are made with dA, of which approximately two are made with the deoxyribose moiety, two are with the N3 of adenine, and two are with the N6 hydrogens. A total of eighteen dispersion contacts are made with dA, of which only one shows interaction with the deoxyribose moiety, sixteen show interaction with the adenine base, and only one shows interaction with the N6 hydrogens.

Once again an extended range of the paratope occurs to accommodate the larger volume of the ssDNA trinucleotide (A-^6m^A-T). The central or primary paratope in this case includes any residues involved in interactions with the d^6m^A moiety. Residues of the primary paratope against d^6m^A moiety include Tyr L48, Glu L49, Lys L52, Leu H102, and Pro H103. Only one H-bond was found with the d^6m^A moiety A-^6m^A-T, in which the carbonyl oxygen of the side-chain of Glu L49 receives the N6 lone hydrogen. A total of five polar contacts are made with the d^6m^A moiety, of which two are made with the adenine base (one to N3 and the other to N5) and three are made with the N6 lone hydrogen. A total of eighteen dispersion contacts are made with the d^6m^A moiety, of which only one or two show interaction with the deoxyribose moiety, fourteen show interaction with adenine base, and six show interaction with the N6 substituents (approximately four contacts to the methyl group and two contacts to the lone hydrogen).

Secondary and tertiary paratopes are present for the nucleotides neighboring the d^6m^A moiety. The residues Ser L55 and Tyr H33 donate a hydrogen to the ^6m^A-T and A-
A phosphate groups, respectively. Only three residues interact with the dA moiety: Tyr H33, Tyr H34 (=Tyr 34), and Trp H101. The phenolic hydroxyl group of Tyr H34 receives a hydrogen from one of the N6 hydrogens of adenine. Twelve dispersion and three polar contacts are made with the dA moiety. Four residues interact with the dT moiety: Ser L55, Tyr H99 (=Tyr 99), Ala H105, and Tyr H106 (=Tyr 106). The side-chain hydroxyl group of Ser L55 receives a hydrogen from the alcohol group of the deoxyribose moiety. Eleven dispersion and three polar contacts are made with the dT moiety.
Figure 34. Predicted paratope interactions of the anti-\(d^{6m}A\) Fv using five flexible residues in the binding pocket with flexible (a) (b) deoxy-\(N^6\)-methyladenosine, (c) (d) unmethylated deoxyadenosine, and (e) (f) ssDNA trinucleotide (A\(^{6m}\)A-T). Black dotted lines indicate H-bonds. Grey spheres indicate hydrophobic interactions and smaller light blue or red spheres indicate that a polar group is involved in either a dispersion or a dipole interaction. The maximal distance from the epitope for a van der Waals contact was the sum of the two radii of the interacting atoms with a scaling factor of 1.3.
The paratopes of the 7C7:C5 Fv binding pocket for each of the three docked antigens (d\(^{6m}\)A, dA, and A\(^{-6m}\)A-T) include the following common residues: Tyr L48, Ser L55, and Pro H103. These three residues are apparently the most critical binding residues. They comprise a large number of the overall contacts, in which Tyr L48 has the most contacts generally followed by Pro H103 and lastly Ser L55. In addition to these residues, leucine residues have been found to make at least a few contacts to the epitope of d\(^{6m}\)A, dA, and A\(^{-6m}\)A-T. Leu H104 makes contacts for the docking of d\(^{6m}\)A and dA to the 7C7:C5 Fv binding pocket, while Leu H102 makes contacts for the docking of d\(^{6m}\)A, dA, and A\(^{-6m}\)A-T. Residues Glu L49 and Lys L52 make at least a few contacts for binding dA around the hydrogens of the N6 position. Coincidentally, Glu L49 and Lys L52 make several contacts on the d\(^{6m}\)A moiety of A\(^{-6m}\)A-T around the methyl group and lone hydrogen of the N6 position.

In fact, the docking of the d\(^{6m}\)A moiety in A\(^{-6m}\)A-T resembles more closely the docking of dA than d\(^{6m}\)A, in which five of the seven residues involved in dA docking are involved in docking of the d\(^{6m}\)A moiety in A\(^{-6m}\)A-T. In fact, the docking of the d\(^{6m}\)A moiety in A\(^{-6m}\)A-T involves only these five paratope residues: Tyr L48, Glu L49, Lys L52, Leu H102, and Pro H103. The complete docking of A\(^{-6m}\)A-T, however, provides interaction with twelve paratope residues versus eleven paratope residues for d\(^{6m}\)A and seven paratope residues for dA. As a result, the overall docking of A\(^{-6m}\)A-T yields more paratope residues directly involved in docking than either d\(^{6m}\)A or dA.

In the docking of d\(^{6m}\)A, residues His L33, Tyr L35, Pro L45, Ile L47, and Leu H104 show interactions with the N6 methyl group. The overall number of interactions with the N6 methyl group of d\(^{6m}\)A alone and d\(^{6m}\)A in A\(^{-6m}\)A-T is thirteen and four,
respectively. The overall number of interactions with the N6 hydrogen of dA that sticks downwards into the binding pocket is four, of which one is a H-bond. Table 9 summarizes the overall number of interactions by category: stacking, H-bond, polar, or dispersion interactions.

Table 9. Summary of the total interactions of the 7C7:C5 Fv with five flexible residues and flexible antigens: d6mA, dA, and ssDNA (A-6mA-T)

<table>
<thead>
<tr>
<th>Type of Interactions</th>
<th>Number of Interactions for Each Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d6mA</td>
</tr>
<tr>
<td>π-Stacking Interactions</td>
<td>0</td>
</tr>
<tr>
<td>Hydrogen Bonds</td>
<td>3</td>
</tr>
<tr>
<td>Polar Contacts</td>
<td>6</td>
</tr>
<tr>
<td>Dispersion Contacts</td>
<td>28</td>
</tr>
</tbody>
</table>

One additional mode of binding for dA that appeared plausible was found from the docking of dA in the binding pocket with the five flexible residues (Figure 35). This binding mode showed dA buried deep into the binding pocket similar to the docking of d6mA; however, dA is in the reverse orientation (a 180° horizontal rotation). The paratope for this alternate binding mode for dA includes the following paratope residues: Pro L45, Tyr L48, Ala L54, Ser L55, Tyr H33, Leu H102, Pro H103, Leu H104, and Ala H105. Eight of these nine paratope residues for binding of dA were also paratope residues predicted in the binding of d6mA, while in the docking model for dA in Figures 33c, d and 34c, d (p. 112, 116), only five of the seven paratope residues in binding of dA were also predicted in the binding of d6mA. The docking model for dA in Figure 35 shows nine polar contacts and twenty-two dispersion contacts. The number of dispersion contacts in the flexible Fv docking model in Figure 35 is identical to the number of
dispersion contacts found in the rigid Fv docking model in Figure 31c, d and 32c, d (p. 102, 106).

Figure 35. Reverse orientation binding mode found in the docking of flexible dA to the 7C7:C5 Fv with five flexible residues in the binding pocket. Top (a) (b) models show the position and orientation of docking of dA in the binding pocket of the 7C7:C5 Fv. Bottom (c) (d) models show the paratope interactions of the 7C7:C5 Fv with dA in a reverse orientation of binding. Black dotted lines indicate H-bonds. Grey spheres indicate hydrophobic interactions and smaller light blue or red spheres indicate that a polar group is involved in either a dispersion or a dipole interaction. The maximal distance from the epitope for a van der Waals contact was the sum of the two radii of the interacting atoms with a scaling factor of 1.3. Color code for the Fv is the same as in Figure 30, p. 100. Atomic color code (grey for carbons and non-polar hydrogens, cyan for polar hydrogens, blue for nitrogen, and red for oxygen) is used for the antigen.

The binding energy for docking of dA in this flexible binding pocket docking model in Figure 35 is -14.22 kcal/mol and the corresponding $K_d$ is $3.757 \times 10^{-11}$ M. As a result, it is more favorable than the one selected in Figures 33c, d and 34c, d (p. 112,
In addition, the contribution from intermolecular energy is much closer to that reported for the selected rigid Fv docking model for dA. The intermolecular energy for the docking model in Figure 35 is -4.82 kcal/mol vs. -4.62 kcal/mol for the rigid Fv docking model in Figures 31c, d and 32c, d (p. 102, 106) vs. -3.59 kcal/mol for the flexible Fv docking model in Figures 33c, d and 34c, d.

Nevertheless, a few inconsistencies exist with the flexible Fv docking model in Figure 35. First, no H-bonds were found to occur in this docking model for dA. Hydrogen bonds are typically important in determining the specificity of anti-ssDNA antibodies. However, three dipole-dipole contacts between the OH groups of Tyr L48, Ser L55, and Tyr H33 and three different polar groups on the dA may require only a slight adjustment in either separation or rotation between paratope and epitope groups to form H-bonds. Second, none of the other antigens suggest this reverse orientation docking mode to occur. Third, the most favorable Fv docking mode of A-^6m-A-T [Figure 33e, f and 34e, f (p. 112, 116)] greatly resembles the reported docking mode for dA in Figures 33c, d and 34e, f. Fourth, slot blot results suggest an affinity difference between binding of d^6mA and dA to the 7C7:C5 Fv of at least six fold, whereas the K_d for the docking model in Figure 35 suggests an affinity difference between binding of d^6mA and dA to the 7C7:C5 Fv of only two fold.

In conclusion, the resulting more favorable energy for docking of dA in Figure 35 may simply be due to the deeper binding of dA into the binding pocket, in which more contacts occur. This docking model hence further supports the predicted binding pocket. In addition, it also supports the hypothesis that the final docked state of dA in the 7C7:C5 Fv is likely to occur deep in the binding pocket as opposed to closer to the surface of the
7C7:C5 Fv. On the other hand, the docking model selected in Figures 33c, d and 34c, d (p. 112, 116) is consistent with the predicted orientation of antigen docking in the 7C7:C5 Fv. The possibility of combining the deeper binding as found in the docking models for d$_{6m}$A in Figures 31a, b-34a, b (p. 102, 106, 112, 116) and in the docking models for dA in Figures 31c, d, 34c, d with the forward orientation (the orientation of the N6 position of d$_{6m}$A or dA towards His L33 and away from Ser L55) seems to represent the most logical prediction for the docking of dA. Nevertheless, the reverse horizontal orientation of dA docking cannot be ruled out. The N6 position for dA contains two polar hydrogens, while the N6 position for d$_{6m}$A contains only one polar hydrogen. This additional polar hydrogen results in the N6 position of dA being more polar than the N6 position of d$_{6m}$A. The His L33 residue easily makes dispersion contacts with the N6 methyl group of d$_{6m}$A, but the corresponding hydrogen of dA sticking down is too small to reach His L33 for interactions. As a result, the positioning of the –$N^6H_2$ group in dA may favor being closer to Ser L55, a polar residue, as shown in Figure 35c, d.

**Conformational Changes in the Five Flexible Residues in the Selected Docking Models**

The five flexible residues (Tyr L35, Ile L47, Tyr L48, Ser L55, and Leu H104) in all of the flexible binding pocket docking models share identical positioning of their C$_\alpha$-C$_\beta$ bonds with their corresponding rigid binding pocket docking models. As a result, the C$_\alpha$-C$_\beta$ bond serves as an unchanging axis for rotation of amino acid side chains in the five flexible residues. In Tables 10-12, conformational changes that compare the five flexible residues in the docking models with these same residues in the corresponding
rigid docking models are listed. Two conformational changes were looked at for docking of d<sup>6m</sup>A, dA, and A-<sup>6m</sup>A-T using ADT-1.5.2.

The first change investigated was the largest change in position of an atom in the flexible residues vs. the same atom in the corresponding rigid residues. This change in position is measured as the largest distance of separation between an atom in the flexible Fv docking models and the same atom in the corresponding rigid Fv docking models.

The second change investigated was the angle of rotation of a side chain group of a flexible residue along the C<sub>α</sub>-C<sub>β</sub> axis relative to the orientation of the same side chain group in the corresponding rigid residue. This rotation was measured by calculating the difference between the dihedral angles of the specified atoms (listed in Tables 10-12) in the flexible residues in the docking models vs. the same atoms in the corresponding rigid residues in the docking models. All angles of rotation were inspected visually to support the accuracy of the calculated angles.

Table 10. Conformational changes of Tyr L35, Ile L47, Tyr L48, Ser L55, and Leu H104 as flexible vs. rigid in anti-d<sup>6m</sup>A docking

<table>
<thead>
<tr>
<th>Residue</th>
<th>Atom, Distance (Å)</th>
<th>Angle of Rotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr L35</td>
<td>H of OH, 0.333</td>
<td>C-C-O-H, 24</td>
</tr>
<tr>
<td>Ile L47</td>
<td>C&lt;sub&gt;δ&lt;/sub&gt;, 0.559</td>
<td>C&lt;sub&gt;α&lt;/sub&gt;-C&lt;sub&gt;δ&lt;/sub&gt;, 17</td>
</tr>
<tr>
<td>Tyr L48</td>
<td>H of OH, 1.945</td>
<td>C-C-O-H, 125</td>
</tr>
<tr>
<td>Ser L55</td>
<td>O of OH, 2.399</td>
<td>C-C-O-H, 176.5</td>
</tr>
<tr>
<td>Leu H104</td>
<td>C&lt;sub&gt;γ&lt;/sub&gt;, 1.205</td>
<td>C&lt;sub&gt;α&lt;/sub&gt;-C&lt;sub&gt;δ&lt;/sub&gt;, NA</td>
</tr>
</tbody>
</table>

*NA = not applicable, due to no changes in the angle of rotation
Table 11. Conformational changes of Tyr L35, Ile L47, Tyr L48, Ser L55, and Leu H104 as flexible vs. rigid in anti-dA docking

<table>
<thead>
<tr>
<th>Residue</th>
<th>Largest Distance of Separation</th>
<th>Angle of Rotation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Atom</td>
<td>Distance (Å)</td>
</tr>
<tr>
<td>Tyr L35</td>
<td>O of OH</td>
<td>0.277</td>
</tr>
<tr>
<td>Ile L47</td>
<td>Cδ</td>
<td>0.320</td>
</tr>
<tr>
<td>Tyr L48</td>
<td>C2 aromatic</td>
<td>3.778</td>
</tr>
<tr>
<td>Ser L55</td>
<td>H of OH</td>
<td>3.312</td>
</tr>
<tr>
<td>Leu H104</td>
<td>Cγ</td>
<td>1.196</td>
</tr>
</tbody>
</table>

Table 12. Conformational changes of Tyr L35, Ile L47, Tyr L48, Ser L55, and Leu H104 as flexible vs. rigid in anti-A-6m A-T docking

<table>
<thead>
<tr>
<th>Residue</th>
<th>Largest Distance of Separation</th>
<th>Angle of Rotation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Atom</td>
<td>Distance (Å)</td>
</tr>
<tr>
<td>Tyr L35</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Ile L47</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Tyr L48</td>
<td>O of OH</td>
<td>1.864</td>
</tr>
<tr>
<td>Ser L55</td>
<td>H of OH</td>
<td>3.313</td>
</tr>
<tr>
<td>Leu H104</td>
<td>Cγ</td>
<td>1.196</td>
</tr>
</tbody>
</table>

The residues Tyr L48 and Ser L55 showed rather large separations and relative rotations between the specified atoms of the flexible vs. rigid binding pocket, while the residues Tyr L35 and Ile L47 showed very small separations and relative rotations if any between the specified atoms of the flexible vs. rigid binding pocket. The larger changes in conformation are primarily in the orientation of the side chain groups. In the case of
Tyr L48, the measured angle of rotation is particularly influenced by the position of the OH group. A rather small separation and rotation occurs in the aromatic ring of Tyr L48 in the flexible binding pocket model for d\textsuperscript{6m}A docking [Figure 34a, b (p. 116)], while a much larger rotation occurs in the aromatic ring of Tyr L48 in the flexible binding pocket model for dA docking (Figure 34c, d).

The change in position of Tyr L48 in the flexible binding pocket is likely to be related to the necessary rotation of the OH group for its involvement in H-bonding with the 5’ oxygen of the deoxyribose moiety in the docking of d\textsuperscript{6m}A. The flexible binding pocket docking model of dA, on the other hand, does not show any H-bond from Tyr L48 because dA docked closer to the surface of the 7C7:C5 Fv, in which this interaction with the 5’ oxygen of the deoxyribose moiety was out of reach. Nevertheless, Tyr L48 of the docked dA in the flexible binding pocket must rotate its ring to maximize contacts with the adenine. This rotation of the phenyl group of Tyr L48 in docking of dA is much larger than the rotation of the phenyl group of Tyr L48 in docking of d\textsuperscript{6m}A. The additional rotation of the phenyl group of Tyr L48 seems to contribute to a more snug fit of the docked dA than would have occurred without this rotation. The rotation of the phenyl group is unnecessary for the docked d\textsuperscript{6m}A because it docked deeper into the binding pocket fitting snug against Tyr L48 as in its starting position (identical position as in rigid Fv docking models).

Ser L55 of the flexible binding pocket docking models showed significant rotations from its starting position. In the case of Ser L55, the change in rotation in docking of d\textsuperscript{6m}A is noticed in the OH group of the side chain, in which the OH group makes nearly a 180° rotation moving it closer towards the surface of the binding pocket.
and further away from the N3 of adenine in d$^{6m}$A. This rotation does not result in any H-bonds involving the OH group of Ser L55, but an H-bond does occur between N3 of adenine and the main chain NH group of Ser L55. As a result, this rotation may help bind d$^{6m}$A deeper into the binding pocket by allowing Ser L55 to make a wider range of contacts with the d$^{6m}$A, including contacts closer to the upper surface of the binding pocket. A similar but smaller rotation is noticed in docking of dA and A-$^{6m}$A-T. In the docking of dA, however, the rotation of the OH group of Ser L55 allowed for Ser L55 to form an H-bond with the alcohol group of the deoxyribose moiety. Similarly, the rotation of the OH group of Ser L55 in docking of A-$^{6m}$A-T allowed for two H-bonds to form: one with the deoxyribose of the dA moiety and one with the neighboring phosphate group.

In the flexible binding pocket docking models, a 1.2 Å shift in the C$\gamma$ atom positions this carbon away from the adenine of d$^{6m}$A, dA, and d$^{6m}$A moiety of A-$^{6m}$A-T. Whether it is nearly a 180° rotation or merely a shifting of the C$\gamma$ atom cannot be determined by analyzing the docking models since the C$\gamma$ is linked to two symmetric methyl groups, which appear to maintain relatively close positions to their initial positions. This shift in position of the C$\gamma$ may allow more room for the adenine of d$^{6m}$A, dA, and d$^{6m}$A moiety of A-$^{6m}$A-T to fit into the binding pocket, but even more so may provide more room for other neighboring residues in the binding pocket, such as His L33, Tyr L35, and Pro H103. An additional possibility is that the formation of a more favorable conformation for Leu H104 occurs upon docking of d$^{6m}$A, dA, or A-$^{6m}$A-T.

The flexible Tyr L35 and Ile L47 show minimal rotations and overall changes from their starting positions. This lack of change in Tyr L35 and Ile L47 is quite logical.
as they are some of the deeper residues of the binding pocket, in which only d\textsuperscript{6m}A docks deep enough to make contacts with these residues. The absence of change in these residues in the A-\textsuperscript{6m}A-T flexible binding pocket docking model is reflective of the inability of the d\textsuperscript{6m}A moiety to dock deep into the binding pocket in this model. In the docking models that show change in Tyr L35 and Ile L47, a minor shift occurs in the position of the phenyl group of Tyr L35 and the C\textsubscript{γ} and C\textsubscript{δ} atoms of Ile L47 that moves them slightly further away from the binding pocket.

In the case of the docking model for d\textsuperscript{6m}A, Tyr L35 and Ile L47 both make contacts with the d\textsuperscript{6m}A. However, the change in position of the OH group of Tyr L35 shifts it slightly closer to the N6 methyl group of d\textsuperscript{6m}A, while the change in position of the C\textsubscript{γ} and C\textsubscript{δ} ethyl group of Ile L47 shifts it slightly further away from the N6 methyl group of d\textsuperscript{6m}A. Similar but smaller changes are noticed in the conformations of the side chains of Tyr L35 and Ile L47 in the flexible binding pocket docking of dA than for the flexible binding pocket docking of d\textsuperscript{6m}A. These changes show no relation to how close they are to the dA or to interactions with the dA, but they may have been influenced by the shifting of the phenyl group of Tyr L48 more towards the adenine of dA.

**Final Analysis of the 7C7:C5 Fv Docking Models and Calculated Binding Energies**

While analyzing the individual conformational changes in the side chains of residues given flexibility in the binding pocket may help to explain why at least some of the rotations occurred in these side chains, this analysis overlooks the quality of the rigid binding pocket docking models. First, the rigid Fv docking models show an overall greater amount of consistency in docking modes between the d\textsuperscript{6m}A, dA, and A-\textsuperscript{6m}A-T.
For instance, all eight of the paratope residues involved in the rigid binding pocket docking of dA are also found in the paratope for docking of d$_{6m}$A, but only five of the seven paratope residues involved in the flexible binding pocket docking of dA are also found in the docking of d$_{6m}$A. In addition, all six of the paratope residues involved in the rigid binding pocket docking of the d$_{6m}$A moiety of A-$^{6m}$A-T are also found in the paratope for docking of d$_{6m}$A, but only three of the five paratope residues involved in the flexible binding pocket docking of the d$_{6m}$A moiety of A-$^{6m}$A-T are also found in the paratope for docking of d$_{6m}$A.

Second, more interactions occur in the rigid Fv docking models for d$_{6m}$A and dA than in the corresponding flexible Fv docking models. These additional interactions lead to more favorable intermolecular binding energies in the rigid Fv docking models. In contrast, a more favorable intermolecular energy occurs in the flexible Fv docking model for A-$^{6m}$A-T than in the rigid Fv docking model for A-$^{6m}$A-T. This energy difference is largely due to an increased number of interactions with the adjacent nucleotides and phosphate groups in the flexible Fv docking model for A-$^{6m}$A-T. A list comparing the intermolecular energies involved in the binding of d$_{6m}$A, dA, and A-$^{6m}$A-T to the 7C7:C5 Fv are reported in Table 13.
Table 13. Intermolecular energies from binding of the 7C7:C5 Fv to d$^{6m}$A, dA, and A-$^{6m}$A-T in the selected rigid and flexible binding pocket docking models

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Intermolecular Energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rigid Binding Pocket</td>
</tr>
<tr>
<td>d$^{6m}$A</td>
<td>-5.5</td>
</tr>
<tr>
<td>dA</td>
<td>-4.62</td>
</tr>
<tr>
<td>A-$^{6m}$A-T</td>
<td>-5.57</td>
</tr>
</tbody>
</table>

Comparison between the rigid and flexible Fv docking models of d$^{6m}$A shows that the two docking models strongly resemble one another in paratope interactions and intermolecular energies of binding. The flexible Fv docking contributes an additional H-bond between the binding pocket and d$^{6m}$A, but shows fewer vdW contacts than the rigid Fv docking of d$^{6m}$A. The ideal scenario would be to maintain all of the vdW contacts found in the rigid Fv docking models and add an additional H-bond between Tyr L48 of the binding pocket and the deoxyribose of d$^{6m}$A or dA. In comparing flexible vs. rigid docking models, the overall changes in the number of interactions are by far the greatest for A-$^{6m}$A-T in favor of the flexible Fv docking model. This change in the number of interactions is easily accounted for by the increase in interactions of the adjacent phosphate groups and nucleotides.

In the rigid Fv docking model for A-$^{6m}$A-T, the thymine is in an awkward position, in which it is turned away from the surface of the Fv. The flexible Fv docking model for A-$^{6m}$A-T shows both of the adjacent nucleotides against the surface of the Fv. In consideration of these additional interactions, the largest change in intermolecular
energy is between flexible and rigid Fv docking models in the docking of A-6m A-T. However, all of the flexible binding pocket docking models appear to overestimate the affinity of the 7C7:C5 Fv for d6m A, dA, and A-6m A-T. On the other hand, the rigid binding pocket docking models appear to underestimate the affinity of the 7C7:C5 Fv for d6m A, dA, and A-6m A-T.

The overestimation of the affinity in the flexible Fv docking models is due to substantial intramolecular energy contributions to the binding energy. These energy contributions are not from interactions within the protein, as much of the changes of the flexible residues in the binding pocket were directly related to association with each of the docked antigens. Some of the internal energy contribution, however, appears to be not due to the association with the docked antigen, but rather a further opening of the binding pocket to more comfortably fit the docked antigen and to allow more favorable conformations in some of the residues of the binding pocket to occur as a result of this opening.

The underestimation of the affinity in the rigid Fv docking models appears to result from two factors. First, an abundant number of vdW interactions were found and generally a few H-bonds. The shear number of overall interactions would normally suggest a higher affinity. For instance, assuming a contribution of -1 kcal/mol per H-bond and a contribution of -0.3 kcal/mol per dispersion interaction in binding, a simple calculation of the number of dispersion interactions and H-bonds in the docking of d6m A reveals that the energy contribution from intermolecular interactions is approximately -13 kcal/mol. A common torsional energy penalty of 1 kcal/mol was found in ADT-1.5.2 that is attributed to torsions and rotations of the antigen. The resulting binding energy
based solely on the number of interactions and these general assumptions is -12 kcal/mol for the docking of $d^{6m}A$ to the 7C7:C5 Fv. Nevertheless, some overlap among residues occurs with vdW contacts and the contribution of -1 kcal/mol in binding is relative to the solution and prior H-bonds of the Fv and antigen before the binding of the antigen to the Fv. Thus, the resulting binding energy may be a few kcal/mol less than the estimated -12 kcal/mol.

A way of adjusting the calculated binding energies from the rigid Fv and flexible Fv docking models is to average the binding energies of the two sets of models for each antigen. These average binding energies would approximate the energies of binding of $d^{6m}A$, dA, and A-$^{6m}A$-T to a binding pocket of two and a half flexible residues. Given that only two main residues (Tyr L48 and Ser L55) show considerable flexibility in the binding pocket of the 7C7:C5 Fv that directly relates to interactions with each antigen, the flexibility of these two residues is quite logical. The additional half flexible residue would apply to minor changes in the remainder of residues in the binding pocket, particularly Pro H103 which showed contacts with adenine of $d^{6m}A$, dA, and d$^{6m}A$ moiety of A-$^{6m}A$-T. Typically, prolines do not show much rotational capabilities, but they are capable of bending flexibility. The averages of the binding energies from the rigid Fv and flexible Fv docking models for each antigen are listed in Table 14. An intermediate $K_d$ for the docking of each antigen to the 7C7:C5 Fv was calculated based on these average binding energies. The binding energies and the corresponding $K_d$ from each set of docking models is also reported in Table 14.
Table 14. Binding energies and inhibitor constants from docking studies with the 7C7:C5 Fv

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Rigid Body Fv Docking</th>
<th>Flexible Residue Fv Docking</th>
<th>Intermediate Docking (Based on the average ΔG of rigid and flexible Fv docking)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Binding Energy (kcal<em>mol⁻¹</em>K⁻¹)</td>
<td>Dissociation Constant, K_d (10⁻³ M)</td>
<td>Binding Energy (kcal<em>mol⁻¹</em>K⁻¹)</td>
</tr>
<tr>
<td>d⁶mA</td>
<td>-5.15</td>
<td>0.169</td>
<td>-14.57</td>
</tr>
<tr>
<td>dA</td>
<td>-3.56</td>
<td>2.46</td>
<td>-13.45</td>
</tr>
<tr>
<td>A⁻⁶mA-T</td>
<td>-2.37</td>
<td>18.2</td>
<td>-14.36</td>
</tr>
</tbody>
</table>
DISCUSSION

Assessment of the 7C7:C5 Fv Docking Models

The sequences for the 7C7:C5 variable genes were obtained and, in turn, the primary structures for the 7C7:C5 variable regions were determined. Based on the primary structures, homology modeling using Modeller9v4 was performed to generate two separate three-dimensional models of the 7C7:C5 Fv. One model Fv was based on crystallographic structures of Fab fragments in an antigen unbound conformation and the other model Fv was based on crystallographic structures of Fab fragments in an antigen bound conformation. While modeling with high identity (80-95% sequence identity) is generally preferred, the idea of modeling based on antigen-bound versus –unbound conformations appears to be relatively novel with surprisingly promising results. Comparison of the antigen-bound-template model Fv versus the unbound-template model Fv revealed a binding pocket which is clearly present in the antigen-bound-template model Fv but not in the unbound-template model Fv. The antigen-bound-template model Fv shows movement of Tyr L48 and Ser L55 and perhaps slighter movements in other spatially neighboring residues resulting in a binding pocket that is open in the antigen-bound-template model Fv but closed off in the unbound-template model Fv.

The “blind docking” approach of predicting binding pockets has shown promising results. In fact, blind docking results using only a single docking job of 100 docking trial runs in AutoDock were used to correctly predict the binding pocket and corresponding ligand binding mode found in crystallographic structures for 34 out of 43 tested proteins. In these 34 proteins, the predicted binding pocket and ligand binding
mode yielded the energy minimum (most favorable energy). In three of the remaining nine cases among the forty-three tested proteins, the crystallographic binding pocket and corresponding ligand binding mode was found as the energy minimum in subsequent docking jobs (experiments) using AutoDock. The last six crystallographic binding pockets and corresponding ligand binding modes were found to rank from 2\textsuperscript{nd} to 7\textsuperscript{th} in terms of most favorable energy relative to the energy minimum.

Thus, to further inspect the validity of the only visually apparent binding pocket, rigid Fv blind docking studies with flexible antigens were done to check areas of antigen docking along the upper V\textsubscript{H} and V\textsubscript{\kappa} interface (interface region in the opposite direction from the amino termini of the C\textsubscript{H}1 and C\textsubscript{\kappa} regions). These studies showed that docking of the antigens d\textsuperscript{6m}A, dA, and d\textsuperscript{6m}A of ssDNA (A-\textsuperscript{6m}A-T) within the predicted binding pocket of the 7C7:C5 Fv gave the most favorable binding energy for both d\textsuperscript{6m}A and dA and the second most favorable binding energy for A-\textsuperscript{6m}A-T. The most favorable conformation found for A-\textsuperscript{6m}A-T did not show the d\textsuperscript{6m}A bound into the binding pocket, but this conformation showed the d\textsuperscript{6m}A moiety near the binding pocket. All K\textsubscript{d} calculations using a rigid body binding pocket of the 7C7:C5 Fv in AutoDock-4.0 were relatively high (10\textsuperscript{-2} to 10\textsuperscript{-4} M).

As a result, an induced-fit model of docking seemed to be more likely between d\textsuperscript{6m}A, dA, and A-\textsuperscript{6m}A-T in determination of K\textsubscript{d}. In support of this hypothesis, other anti-ssDNA binding domains were found to undergo induced-fit conformational changes that allow for contacts to the buried nucleic acid to be maximized\textsuperscript{42,49-50,82}. Accordingly, five flexible residues within the predicted 7C7:C5 binding pocket showed rotational capabilities and, thus, seemed like a good starting point for docking studies using an
induced-fit binding model. The docking studies with the five flexible binding pocket residues produced conformations with $K_d$ of $10^{-10}$ to $10^{-11}$ M, which show a $10^7$ to $10^9$ fold higher affinity than the rigid body binding pocket docking studies.

Comparison of the intermolecular energy between d6mA docking to the rigid body binding pocket and to the flexible residue binding pocket is almost identical, in which intermolecular interactions are actually favored in the rigid body binding pocket by -0.06 kcal/mol. The primary source of this change in $K_d$ happens to occur through the contribution of internal energy or energy generally associated with the conformation of the ligand involved in binding. Upon making residues flexible in the binding pocket, however, the internal energy increased substantially more than what is common for ligand internal energy alone.

The primary source of the increase in internal energy was from energy that resulted from changes in the conformations of the flexible binding pocket residues. These changes in residue conformations were found in some cases to maximize interactions between the Fv and the docked antigen. In other cases, these conformational changes simply opened the binding pocket slightly more giving more room for residues within this area to take on more favorable conformations and for interactions of the antigen with deeper residues of the binding pocket to be more accessible. Typically, the internal energy term is close to zero in docking studies, but these studies used rigid body proteins. Furthermore, the appearance of an internal energy term is often associated with the desirable effect of placing top ranked binding mode clusters with more favorable binding energies.
For instance, occasionally, top ranked binding modes (or binding modes with no unacceptable clashes or unusual conformations) will result in a calculated low free energy. The internal energy term in AutoDock-4.0 is primarily a result of energy differences in the protein before and after ligand docking. This energy contribution that results from conformational changes in the binding pocket of the protein is primarily overlooked in rigid protein docking. Addition of internal energy to calculated free energy has been found to help boost the free energy of appropriate docking modes in accordance with their conformational rankings.

Nevertheless, these changes in internal energy help to more accurately mimic “induced-fit” binding, in which residues of the protein are actually in a state of conformational flux during the binding process. This state of flux is dynamic and is brought on by interaction with the ligand, in which interaction with the ligand may spatially push to enhance interactions within the protein itself. Such secondary interactions may or may not be associated with multivalency, in which binding of a primary ligand opens a secondary pocket in the protein for binding of a secondary ligand.

Figure 36 shows the primary binding pocket of d\textsuperscript{6m}A and a possible secondary binding pocket adjacent to it on the left that may further open upon d\textsuperscript{6m}A binding and, consequently, accept a second d\textsuperscript{6m}A or possibly even the dA moiety adjacent to the d\textsuperscript{6m}A moiety of A\textsuperscript{6m}A-T. Interestingly, in Figures 32e, f and 34e, f (p. 106, 116), the dA moiety of A\textsuperscript{6m}A-T contacts the surface of the 7C7:C5 Fv near this possible secondary binding pocket in both rigid and flexible Fv docking models. Of the highlighted residues in Figure 36, Tyr H33 belongs to CDR-H1 and Tyr H99 and Tyr H106 belong to CDR-H3. Tyr H27 and Arg H98 are considered framework residues.
Figure 36. Possible secondary binding pocket on the left of the primary binding pocket of the 7C7:C5 Fv. Residues selected or highlighted for this secondary binding pocket include Tyr H27, Tyr H33, Arg H98, Tyr H99, and Tyr H106.

Admittedly, treatment of ligand receptor residues as flexible can result in large changes in $K_d$, particularly due to the exponential relationship between $\Delta G$ and $K_d^{165-166}$ and, thus, their usage should be considered cautiously. Four flexible protein binding site residues of thyroid hormone receptor $\beta$ (TR$\beta$) were used in a study towards calculation of a binding free-energy of -11.78 kcal/mol using AutoDock 4.0 $^{167}$. No strategy was reported for scaling the internal energy contribution in the study by Du et al $^{167}$. Interestingly, the interacting surface area was predominantly hydrophobic, which happens to be the same circumstance for interaction of the 7C7:C5 Fv with $d^{6m}A$ and related antigens. The 7C7:C5 Fv also happens to form two main chain H-bonds with $d^{6m}A$ and dA. Such H-bonds are associated with firm anchoring of the protein to the
interacting ligand and with conformational stabilization that directs the induced-fit \(^\text{168-169}\). As a result, use of flexible residues around the 7C7:C5 Fv binding pocket did not seem unreasonable.

Ultimately, the use of flexible residues in a protein model is a choice of the primary researcher. In this case, the calculated \(\Delta G\) and corresponding \(K_d\) of antigen interactions with the rigid body Fv were all too unfavorable to be normal for the Fv of a MAb produced specifically against \(d^{6m}A\) through hybridoma technology. As a result, five flexible residues were incorporated into the binding pocket of the Fv. The resulting binding modes gave \(K_d\) of \(10^{-10}\) M to \(10^{-11}\) M that seem to be at least slightly below normal for the range of anti-nucleic acid binding (\(K_d\) of \(10^{-7}\) M to \(10^{-10}\) M) found in the literature \(^{55-56, 170-171}\).

In the tutorial for using AutoDock-4.0 with ADT-1.5.2, only two macromolecule (protein) residues were selected for flexibility producing an additional six active torsions (six rotatable bonds) to the total number of active torsions involved in ligand binding \(^{157}\). The five residues selected to be flexible in the 7C7:C5 binding pocket produced twelve additional active torsions, allowing for approximately double the flexibility of the binding pocket of the protein in the tutorial. As a result, the number of flexible residues applied to the binding pocket in the tutorial suggests a possible overuse of flexibility in the binding pocket of the 7C7:C5 Fv. Additionally, the resulting \(\Delta G\) and \(K_d\) of the \(d^{6m}A\) and related antigen binding modes to the 7C7:C5 Fv suggests an overuse of flexibility in the binding modes. Lastly, evidence from biological phenomena such as the catalysis of methyl group transfer from SAM to adenine in DNA by \(M.TaqI\) involves two critical
residues (an ASN and a PRO) for the transfer process. These two residues involve interactions on opposite sides of the adenine base in DNA.

Conformational flexibility for such transfer processes can be critical and, thus, modeling of such processes should involve setting the primary interacting residues to be flexible. In light of this knowledge, the docking models of $d^{6m}A$ and related antigens to the 7C7:C5 Fv would likely have produced a more accurate $\Delta G$ and $K_d$, if only TYR L48 and SER L55 of the three main binding residues (TYR L48, SER L55, and Pro H103) were given flexibility. These residues are considered the main binding residues simply because they make the majority of interactions with each of the three antigens ($d^{6m}A$, dA, and A-$6m$A-T) used in this study. Prolines are generally treated as rigid in modeling simply because they tend to lack rotatable bond flexibility in its side-chain as a result of being a five-membered ring, in which any flexibility generally occurs in the form of folding or bending flexibility as opposed to rotatable bond torsions.

One possible solution for $\Delta G$ and $K_d$ being too high (unfavorable) in the rigid binding pocket docking and too low (favorable) in the flexible residue binding pocket docking is to calculate an average of the $\Delta G$ values from the docking experiments of the rigid Fv and the corresponding $\Delta G$ values from the docking experiments of the Fv with flexible residues. The average $\Delta G$ could then be used to calculate an intermediate $K_d$. The 7C7:C5 Fv in solution may experience an intermediate amount of flexibility in between the rigid and flexible residue docking models shown in the current study. As a result, calculation of intermediate $\Delta G$ and $K_d$ makes the most sense in the given circumstance. The calculated intermediate (based on an average of the obtained $\Delta G$ value from rigid and analogous flexible docking models) $\Delta G$ and corresponding $K_d$ for
$d^{6m}A$, dA, and A$^{-6m}$-T are -9.86 kcal/mol and $5.92 \times 10^{-8}$ M, -8.505 kcal/mol and $5.831 \times 10^{-7}$ M, and -8.365 kcal/mol and $7.835 \times 10^{-7}$ M, respectively. All of these calculated intermediate values for $\Delta G$ and $K_d$ seem quite reasonable for binding of $d^{6m}A$, dA, and A$^{-6m}$-T. Given these intermediate values for $K_d$, the binding affinity of the 7C7:C5 Fv against $d^{6m}A$ is approximately ten fold more favorable than it is against dA and thirteen fold more favorable than it is against A$^{-6m}$-A-T.

**Comparison of Docking using Rigid Body 7C7:C5 Fv with Flexible Antigen vs. 7C7:C5 Fv with Five Flexible Residues and Flexible Antigen**

The docking model comparisons between the 7C7:C5 Fv with a rigid binding pocket versus the 7C7:C5 Fv with flexible residues in its binding pocket suggest that if the 7C7:C5 binding pocket favors flexibility in the binding of $d^{6m}A$, dA, and A$^{-6m}$-T, then a more apparent difference in the binding of $d^{6m}A$ versus dA and $d^{6m}A$ moiety of A$^{-6m}$-A-T would occur. Given the sheer similarity of structure among the tested antigens, the difference in the final docked state should be more subtle between $d^{6m}A$ and dA than the results from the flexible Fv docking models. This subtle difference seems accurately represented by the rigid Fv docking models, in which the binding modes of the adenine ring system and deoxyribose moieties are nearly identical for $d^{6m}A$ and dA. In fact, the main difference in the mode of $d^{6m}A$ vs. dA is that approximately eleven additional vdW interactions are made with the N6 methyl group of $d^{6m}A$ than the corresponding N6 hydrogen of dA. Comparison of the $K_d$ between $d^{6m}A$ and dA in the rigid Fv docking models reveals that docking of $d^{6m}A$ produces a $K_d$ that is approximately six fold less than the $K_d$ for docking of dA. This same difference in affinity was demonstrated to occur experimentally by slot blot.
After careful analysis of the two sets of docking models, the evidence strongly favors the rigid Fv docking models as being closer to the actual final docked state for at least two of the three tested antigens (dA and A-6mA-T). The major significant difference between rigid and flexible Fv docking models is the depth of binding for dA and d6mA moiety of A-6mA-T, in which the rigid Fv docking models show deeper docking of dA and A-6mA-T. The depth of docking of dA is analogous to the depth of docking of d6mA. The d6mA moiety in A-6mA-T in the rigid Fv docking models appears to bury as deep into the binding pocket as it can proceed, which is not the case for the d6mA moiety in the flexible Fv docking models. In the case of flexible Fv docking, the d6mA moiety of A-6mA-T appears to dock at more of a diagonal orientation similar to the docking of dA. In the rigid Fv docking models, the docking of d6mA, dA, and d6mA moiety into the binding pocket of the 7C7:C5 Fv occurs in a nearly vertical position for these antigens.

However, even in the rigid Fv docking models, the docking of the d6mA moiety of A-6mA-T is not as deep as the docking of d6mA or dA alone due to the additional nucleotides and phosphates covalently linked to the d6mA moiety, which place steric restrictions on the movement of the d6mA moiety into the binding pocket. These additional nucleotides and phosphates result in surface interactions with the Fv outside of the primary binding pocket. Many of these additional interactions serve in a compensatory manner for the d6mA moiety not being able to bind as deep as d6mA alone. The flexible Fv docking of A-6mA-T appears to more accurately represent the binding contribution from interactions of the nucleotides adjacent to the d6mA moiety in terms of their positioning towards the Fv surface. In the rigid Fv docking of A-6mA-T, the dT moiety is awkwardly turned up and away from the Fv surface.
In both rigid Fv and flexible Fv docking models, $d^{6m}A$ binds deep into the binding pocket of the 7C7:C5 Fv and, thus, the binding modes appear quite similar with perhaps a more induced-fit appearance in the flexible Fv docking model, in which rotational ability of the phenolic hydroxyl group of Tyr L48 allows it to act as a hydrogen bond donor to the alcohol group of the deoxyribose moiety. Nevertheless, the number of overall vdW interactions slightly favors the rigid Fv docking model. Total interactions found with the N6 methyl group were equal for the rigid and flexible Fv docking models. By combining information from the rigid and flexible Fv docking models, the docking of $d^{6m}A$ quite possibly incorporates all of the interactions found in the rigid Fv docking model with an additional H-bond from the flexible Fv docking model. This additional H-bond only required minor aspects of flexibility, such as rotation of the phenolic hydroxyl group of Tyr L48 towards the alcohol group of the deoxyribose moiety.

Upon further analysis of the flexible Fv docking models for docking of dA and A-$^{6m}$A-T, these models were found to possibly represent an initial/transitional docking state for $d^{6m}A$ and related antigens. In support of this hypothesis, the binding modes of dA and $d^{6m}A$ moiety of A-$^{6m}$A-T in the flexible Fv binding pocket show dA and $d^{6m}A$ moiety in significant interactions with many upper surface residues of the binding pocket such as Glu L49 and Lys L52. Specifically, these interactions with Glu L49 and Lys L52 of the paratope occur at the N6 position of A and $^{6m}$A, in which vdW contacts and a H-bond is made. Glu L49 and Lys L52 make many more contacts at the N6 position to the $d^{6m}A$ moiety of A-$^{6m}$A-T than to dA alone. The majority of these hydrophobic contacts are made with the N6 methyl group. In the case of the $d^{6m}A$ moiety, Glu L49 H-bonds with the N6 lone hydrogen that faces up towards the binding pocket exit. This H-bond, in
turn, results in the initial entrance of the N6 methyl group into the binding pocket. This interaction suggests that the $d^{6m}$A moiety initially binds slightly deeper in the binding pocket than dA because the H-bond occurs from the N6 hydrogen sticking upwards as opposed to the N6 hydrogen sticking downwards as occurs in dA.

Additionally, the docking of dA and $d^{6m}$A moiety occurred in more horizontally slanted positions in the flexible Fv binding pocket than in the rigid Fv binding pocket. These positions allowed for Ser L55 to H-bond with the alcohol group of the deoxyribose moiety on approximately the opposite vertical end from the Glu L49 H-bond. The initial tucking of the N6 methyl group downwards into the binding pocket by particularly Glu L49 results in active pulling of the N6 methyl group and, in turn, the adenine base deeper into the binding pocket. This active pulling may result from surface contacts that essentially push the N6 position and bottom of the adenine ring down into the binding pocket.

One driving force for this active system may be that the number of favorable contacts increases as the methyl group and the adenine base move deeper into the binding pocket. From the other vertical end of the $d^{6m}$A moiety, the Ser L55 acts as a “closing gate” to push the $d^{6m}$A moiety from the front against the rear of the binding pocket resulting in the $d^{6m}$A moiety moving in a vertical rotation deeper into the binding pocket. As the rotation of $d^{6m}$A moiety into the binding pocket proceeds it contacts Tyr L48 with its edges rotating it more towards face to face contacts. The pressure from contacting Tyr L48, in turn, forces pressure against Pro H103 resulting in slight bending of Pro H103. Thus, Tyr L48 acts like one side of a clamp and Pro H103 acts like a spring moiety. Given the similarity in structure of $d^{6m}$A, dA, and $d^{6m}$A moiety, the mechanism of
binding should be nearly identical. As a result, the predicted mechanism of binding of 
\(d^{6m}A, dA, \text{ and } d^{6m}A\) is shown in Figure 37.

![Diagram showing the predicted mechanism of binding of deoxy-N\(^6\)-methyladenosine and related antigens to the 7C7:C5 Fv binding pocket. Only critical residues that directly direct the binding of \(d^{6m}A\) were included in the shown mechanism. Orange dotted arrows indicate direction of horizontal rotation, blue dotted arrows indicate direction of compression, purple arrows indicate breaking of an interaction, and long black arrows indicate a progression of \(d^{6m}A\) towards its final binding state. H-bonds are represented as black dotted lines. R groups with black wiggly lines indicate further amino acid linkages. \(R^1\) groups indicate both carboxyl and amide groups in a peptide bond that links to further amino acids.]

Figure 37. Predicted mechanism for the mode of binding of deoxy-\(N^6\)-methyladenosine and related antigens to the 7C7:C5 Fv binding pocket. Only critical residues that direct the binding of \(d^{6m}A\) were included in the shown mechanism. Orange dotted arrows indicate direction of horizontal rotation, blue dotted arrows indicate direction of compression, purple arrows indicate breaking of an interaction, and long black arrows indicate a progression of \(d^{6m}A\) towards its final binding state. H-bonds are represented as black dotted lines. R groups with black wiggly lines indicate further amino acid linkages. \(R^1\) groups indicate both carboxyl and amide groups in a peptide bond that links to further amino acids.
Structural Features of the 7C7:C5 Fv Docking Models

All of the docking models for the 7C7:C5 Fv show significant contribution from an aromatic amino acid (Tyr L48) involved in H-bonding and several vdW interactions, a polar amino acid (Ser L55) involved in H-bonding and a few vdW interactions, and a non-polar amino acid (Pro H103) involved in a couple of vdW interactions. Interestingly, these residues match up quite closely with the conserved catalytic motif IV of N-DNA MTs, in which the residues are Asn/Asp/Ser-Pro-Pro-Tyr/Phe\(^\text{23}\). Slashes indicate either...or and dashes indicate linkages.

Key paratope residues of the 7C7:C5 Fv also match up quite closely with the adenine binding pocket in the active site of aminoglycoside phosphotransferase enzyme (APH(3')-IIIa)\(^\text{72}\). Three key residues (a tyrosine, a serine, and an alanine) for binding adenine occur in this active site, of which all three of these residue types occur in the 7C7:C5 Fv paratope. Furthermore, these residues serve to provide similar interactions in all three of the mentioned adenine binding domains. For instance, the Asn of motif IV of M.TaqI (an adenine MT)\(^\text{23}\), the active site Ser of APH(3')-IIIa\(^\text{72}\), and Ser L55 of the 7C7:C5 Fv are all examples of polar amino acids that provide important H-bonds in interacting with adenine. In docking of d\(^6\)mA and dA to the 7C7:C5 Fv, this H-bond is made to N3 of the adenine base. Additionally, the vdW contacts from Ser L55 are made primarily to the middle rear edge (edge slightly above the N3 position of adenine) of the adenine base in d\(^6\)mA and dA.

Of the motif IV residues in M.TaqI, the Tyr residue of motif IV is found to engage in a \(\pi\)-stacking interaction and a H-bond on one face of the adenine base and the Asn and adjacent Pro residue of motif IV are aligned along the other face of the adenine
base, in which both of these residues contribute H-bonds along with likely vdW interactions. The remaining Pro residue of motif IV may be involved in vdW interactions along the edge of the five membered ring of adenine. Although no Phe residues were found in the interaction of the 7C7:C5 Fv with da or dA, one Phe residue (not part of motif IV) of M.TaqI was involved in an edge-to-face \( \pi \) interaction along the N3-N4 of adenine.

Similar to Tyr L48 of the binding pocket of the 7C7:C5 Fv, the Tyr residue of the active site of APH(3')-IIIa engages in a face-to-face \( \pi \)-stacking interaction with the six-membered ring of the adenine base. Nevertheless, in the case of the 7C7:C5 Fv paratope interactions with the adenine base of da, da, and da moiety of A-6mA-T, Tyr L48 is only involved in a \( \pi \)-stacking interaction with the da moiety of A-6mA-T and additionally makes several hydrophobic contacts to this moiety. Tyr L48 was not found to engage in a H-bond with the adenine base of the da moiety of A-6mA-T. Similarly, the Tyr of the active site of APH(3')-IIIa was not found to engage in a H-bond with the adenine base.

However, the possibility of the phenolic hydroxyl group of Tyr L48 H-bonding to the N3 position in the docking of da moiety of A-6mA-T does exist. Such a H-bond would only require rotation of the phenolic hydroxyl group towards the N3 position. Unlike docking of the da moiety of A-6mA-T, Tyr L48 was involved in a H-bond with the deoxyribose moiety of da alone. While da did not show a H-bond between the deoxyribose moiety and Tyr L48 in its flexible Fv docking model, this docking model seemed to represent transitional states of binding and, thus, its final docked state would more than likely also contain such a H-bond from Tyr L48. The da moiety of A-6mA-
T, on the other hand, does not appear to be in range for a H-bond between its deoxyribose moiety and Tyr L48 due to the involvement of Tyr L48 in a stacking interaction with the lower portion of the six-membered ring of adenine.

On the other side of the six-membered adenine ring in d$^{6m}$A, dA, and d$^{6m}$A moiety of A$^{-6m}$A-T, Pro H103 and at least one CDR-H3 Leu residue provided the primary source of hydrophobic contacts. One possibility for the lack of π-stacking interactions from Tyr L48 in the docking of d$^{6m}$A and dA is that the residues interacting with the adenine base on the other side are incapable of stacking interactions because they are not flat and, thus, cause misalignment of the adenine base for such flat interactions with Tyr L48. Nevertheless, in the docking of A$^{-6m}$A-T the covalent linkages to phosphates and, in turn, the linkages of the phosphates to other nucleotides helps to restrict the d$^{6m}$A moiety enough to allow the six-membered ring of adenine to align in a face to face planar arrangement with Tyr L48 resulting in a π-stacking interaction.

All crystal structures of anti-poly(dT) or dTdC Fabs thus far have shown at least one π-stacking interaction involving a tyrosine paratope residue with each thymine or cytosine base $^{42,49-50,82}$. The influence of the neighboring phosphate and nucleotide linkages, respectively, on π-stacking interactions has yet to be determined. The crystallographic structure of an anti-monomonucleoside or a mononucleotide with the appropriate mononucleoside or mononucleotide bound would have to be obtained to determine if any π-stacking interactions actually occur. In addition, both thymine and cytosine are pyrimidines and, thus, are six-membered aromatic rings versus the aromatic five- and six-membered conjoined rings of adenine. Thus, congruency of the aromatic rings may be an important aspect for stacking interactions.
Furthermore, in M.TaqI, the Tyr of motif IV contributes its stacking interaction with the lower bottom of the six-membered ring of adenine in DNA. Similarly, the rigid Fv docking model showed that Tyr L48 of the 7C7:C5 Fv contributes its stacking interaction with the lower bottom of the six-membered ring of adenine in ssDNA. As a result, despite the aromaticity of the five-membered ring in adenine through hyperconjugation, stacking interactions with tyrosine residues thus far appear to be limited to the six-membered ring of adenine. This factor alone may make it easier for a thymine or cytosine to align with tyrosine than adenine in mononucleoside or mononucleotidem form and could help to explain why docking of the d$_{6m}$A or dA alone did not result in a π-stacking interaction.

**Summary of the 7C7:C5 Fv Binding Pocket and Paratopes**

The 7C7:C5 Fv primary binding pocket is located along the upper (opposite direction from the constant region), frontal V$_{H}$ (left side) and V$_{K}$ (right side) interface. The primary binding pocket is located at the interface between CDR-H3 and CDR-L2 and only involves these two out of the six CDRs. A couple of framework residues also comprise the binding pocket. These residues include Tyr L35, Pro L45, Ile L47, and Tyr L48, of which all four interact directly with d$_{6m}$A. Framework residues, especially FR residues that flank CDRs, of other Fvs have been previously found to directly interact with epitopes. A hypothetical secondary binding pocket shown in Figure 36, p. 136, may open further during docking of the d$_{6m}$A moiety of A-$_{6m}$A-T into the primary binding pocket, in which the dA moiety may be allowed to dock into this secondary binding pocket that is directly to the left of the primary binding pocket.
The 7C7:C5 Fv paratope is essentially a suborder of specific residues in the 7C7:C5 Fv binding pocket, in which the number of residues directly involved in antigen-binding depends on the specific antigen docked into the binding pocket. Based on the collective information of all the docking models of the 7C7:C5 Fv reported herein, the predicted 7C7:C5 Fv anti-d\textsuperscript{6m}A paratope includes the following eleven residues: His L33, Tyr L35, Pro L45, Ile L47, Tyr L48, Ala L54, Ser L55, Leu H102, Pro H103, Leu H104, and Ala H105. Of these eleven residues, three (His L33, Tyr L35, and Ile L47) are missing from the paratope against dA as a result of a hydrogen in place of a methyl group in the N6 position.

Accordingly, the methyl group in the N6 position is favored over the corresponding hydrogen by approximately eleven vdW contacts. His L33, Tyr L35, and Ile L47 contribute a total of eight vdW contacts to d\textsuperscript{6m}A all of which are in the vicinity of the N6 methyl group. As a result, the main difference in binding affinity is attributed to the larger size of the N6 methyl group than the corresponding N6 hydrogen. This size difference allows the N6 methyl group to not only fit deeper into the binding pocket, but also to provide a greater surface volume for making vdW contacts than a N6 lone hydrogen.

Ser L55 and Leu H102 contribute to main-chain H-bonding of the 7C7:C5 Fv with d\textsuperscript{6m}A and dA. This H-bonding is believed to be nucleotide base-specific, in which the N3 and N6 positions of adenine are involved in the H-bonding. In addition, these H-bonds are believed to be of the utmost importance in d\textsuperscript{6m}A and dA arriving at their final docked conformations in the 7C7:C5 Fv binding pocket. Tyr L48 also contributes an H-bond requiring the rotation of the phenolic hydroxyl group towards the 5’ oxygen of the
deoxyribose moiety in \( d^{6m} A \) and \( dA \). This H-bond is not base-specific, but contributes to maintaining \( d^{6m} A \) and \( dA \) firmly into the 7C7:C5 Fv binding pocket. More importantly, Tyr L48 is by far the largest contributor of hydrophobic contacts.

No \( \pi \)-stacking interactions were found to occur with the lone mononucleotide forms of \( d^{6m} A \) and \( dA \); however, one \( \pi \)-stacking interaction was found to occur with the \( d^{6m} A \) moiety of A-\(^{6m}A\)-T. This interaction may be possible only because of orientation restrictions placed on the \( d^{6m} A \) moiety via the covalent linkages to phosphate groups and, in turn, covalent linkages of the phosphate groups to the corresponding adjacent nucleotides. While these additional linkages and adjacent nucleotides may help to contribute to stacking of Tyr L48 with the six-membered ring of adenine of the \( d^{6m} A \) moiety, they also appear to prevent or at least perhaps redistribute other important interactions. In the docking model in which the Tyr L48 stacking interaction was found with the \( d^{6m} A \) moiety of A-\(^{6m}A\)-T, no H-bonds were made with the \( d^{6m} A \) moiety of A-\(^{6m}A\)-T.

Hypothetically, these H-bonds with minor rearrangement could be redistributed to interact with the phosphate groups as shown in the flexible Fv docking model for A-\(^{6m}A\)-T or with the adjacent nucleotides as shown in the rigid and flexible Fv docking models for A-\(^{6m}A\)-T. Such interactions with the phosphate groups and adjacent nucleotides function as compensatory interactions in place of those interactions that were prevented by linkages to the phosphate groups and adjacent nucleotides. Additionally, it seems rather unlikely that at least some of the residues that H-bond with \( d^{6m} A \) would not also H-bond with the \( d^{6m} A \) moiety of A-\(^{6m}A\)-T. Under this assumption, the H-bonds from residues like Tyr L48, Ser L55, and Leu H102 may simply be redirected to other areas of
the epitope of A-[^6m]A-T than are present in docking of d[^6m]A alone. For instance, looking at the rigid Fv docking model for A-[^6m]A-T, approximately a 90° rotation of the phenolic hydroxyl group of Tyr L48 would bring this group in the proper orientation to H-bond to the N3 position of the adenine ring of the d[^6m]A moiety.

In summary of the interactions involved in docking of d[^6m]A and related antigens to the 7C7:C5 Fv, three H-bonds were found to likely occur with d[^6m]A and dA and a few H-bonds may or may not also occur with d[^6m]A moiety of A-[^6m]A-T; five to six, six, and one to five polar contacts occur with d[^6m]A, dA, and d[^6m]A moiety of A-[^6m]A-T, respectively; and, lastly, 28-32, 18-22, and 16-18 non-stacking hydrophobic contacts occur with d[^6m]A, dA, and d[^6m]A moiety of A-[^6m]A-T, respectively. Only one π-stacking interaction occurred with the d[^6m]A moiety of A-[^6m]A-T. No stacking interactions were found for d[^6m]A or dA.

**Implications for the Potential Uses of the 7C7:C5 Fv**

A monoclonal antibody, the 7C7:C5 MAb, was previously produced against N[^6]-methyladenosine[^96], a naturally occurring methylated nucleotide presently known to occur in prokaryotic and eukaryotic DNA and in viral and eukaryotic RNA[^1,7]. This MAb was demonstrated to interact with higher affinity for d[^6m]A than dA or d[^5m]C. While the 7C7:C5 MAb showed cross-reactivity to dA, the affinity was approximated by slot blot to be six fold greater for d[^6m]A than for dA. In the case of the competition ELISAs[^96], insignificant cross-reactivity to dATP and d[^5m]CTP was noticed mainly because the amount of CV NY-2A genomic DNA (50 ng) used in the solid phase was below the normal limits of detection for these antigens. By slot blot, the limits of detection for a
strand of pUC-19 DNA (2686 bases) was determined to be around 200 ng, while the limit of detection of a strand of pUC-19 DNA with a single $d^6mA$ was determined to be 100 ng and below. Furthermore, detection of CV NY-2A genomic DNA while only at 50 ng contains many $d^6mA$ and, thus, was easily detected by ELISA $^{96}$.

The 7C7:C5 MAb may have higher affinity for $d^6mA$ in ssDNA than $d^6mA$ in dsDNA. This specificity would be unlikely to be a result of biochemical differences between $d^6mA$ in dsDNA versus $d^6mA$ in ssDNA, but rather it would likely be due to conformational restrictions. In dsDNA, adenosine, particularly the N6 position of adenosine, is facing the interior of the structure and, thus, may not be as accessible as adenosine in ssDNA since removal of the second strand of DNA results in adenosine being open for interaction. In immunochemical blots, the $-N^6HCH_3$ of $d^6mA$ simply yields a stronger stain than the $-N^6H_2$ of dA, suggesting that this area of $6mA$ is significant for tighter binding. However, the appearance of stain for unmethylated DNA suggests that some interaction must occur with the rings of adenine and possibly the phosphodeoxyribose backbone of DNA as well.

The binding mode of $d^6mA$, dA, and A-$^6mA$-T was inferred to be of an intermediate nature between the rigid and flexible Fv docking models with the primary interactions demonstrated by the rigid Fv docking models and additional interactions represented by the flexible Fv docking model for $d^6mA$. Such an intermediate binding mode has been suggested previously, in which movement or rotation of residues primarily occurs in rigid groups $^{82}$. As a result, an intermediate $K_d$ was calculated based on the average $\Delta G$ of binding between the rigid Fv and flexible Fv docking models for each docked antigen. The $K_d$ values for each antigen docked to the 7C7:C5 Fv were as
follows: 5.92 x 10^{-8} \text{ M}, 5.831 \times 10^{-7} \text{ M}, and 7.835 \times 10^{-7} \text{ M} for the docking of d_{6m} A, dA, and A-{6m} A-T, respectively. These K_d equate to approximately a ten and thirteen fold greater affinity for d_{6m} A than dA and A-{6m} A-T, respectively.

While an experimental K_d or K_a has yet to be determined for the binding of d_{6m} A moiety in ssDNA to the 7C7:C5 Fv, the K_d appears to be directly influenced by the depth of binding of the d_{6m} A moiety into the binding pocket of the 7C7:C5 Fv. The depth of binding in the selected docking models of A-{6m} A-T to the the 7C7:C5 Fv suggests that the K_d of 7.835 \times 10^{-7} \text{ M} is reflective of a maximum dissociation (minimum affinity) of binding, as keeping the d_{6m} A moiety any further out of the binding pocket seems illogical. In addition, AutoDock-4.0 did not appear to adequately maximize interactions of the two neighboring nucleotides to the d_{6m} A moiety of A-{6m} A-T, particularly in the rigid Fv docking model. As a result, the correct K_d for docking of A-{6m} A-T to the 7C7:C5 Fv may range in between 7.835 \times 10^{-7} \text{ M} and 5.92 \times 10^{-8} \text{ M}. Lastly, whether or not any synergistic or obstructive effects occur in the binding of d_{6m} A in ssDNA to the complete MAb, which are not present in the binding of d_{6m} A in ssDNA to the Fv alone, has yet to be analyzed.

From a practical standpoint, the affinity for the d_{6m} A moiety of A-{6m} A-T is of primary concern, as this form of d_{6m} A occurs in single-stranded DNA. Based on the experimental K_d and the current calculated K_d of the 7C7:C5 Fv for A-{5m} A-T, the use of the 7C7:C5 Fv or MAb would be on the weaker affinity side for therapeutic use. Thus, its current uses should primarily be limited to genotypic or diagnostic detection methods. An affinity enhancement via point mutations could be done to improve the 7C7:C5 Fv affinity against d_{6m} A in DNA to 10^9 \text{ M}^{-1}, which is the desired affinity for therapeutic
uses. A study with MAbs (ED-10 and ED-84) against unmethylated DNA found a $K_a$ of $10^9 - 10^{10}$ M$^{-1}$, confirming that such high affinities are possible between MAbs and DNA$^{55}$.

The use of the 7C7:C5 Fv or MAb as a diagnostic tool and perhaps the use of an affinity enhanced version of the 7C7:C5 Fv or MAb as a therapeutic tool requires answering two primary questions first. 1) What is the primary cross-reactive antigen for the 7C7:C5 Fv? 2) What is the difference in binding affinity of the primary antigen and the primary cross-reactive antigen against the 7C7:C5 Fv? Adenosine by far bears the closest structural resemblance to $6m \text{A}_{os}$, the antigen used to develop the anti-$6m \text{A}_{os}$ MAb, among the other possible ribonucleosides and naturally occurring methylated ribonucleosides. As a result, $\text{A}_{os}$ is most likely to bind to the anti-$6m \text{A}_{os}$ MAb with higher affinity than other ribonucleosides such as: $5 \text{mC}_{os}$, $N^4 \text{mC}_{os}$, guanosine ($G_{os}$), or $N^7 \text{mG}_{os}$). Therefore, only the docking of $d\text{A}$ was modeled as a control for comparison to the docking of $d^{6m}\text{A}$.

Based on rigid Fv docking models, $d^{6m}\text{A}$ was found to bind to the 7C7:C5 Fv with six fold greater affinity than $d\text{A}$. This affinity difference supports the difference in affinity approximated by slot blot. The difference in affinity based on average $\Delta G$ values between rigid and flexible Fv docking models was ten fold in favor of $d^{6m}\text{A}$ over $d\text{A}$. All experimental evidence thus far suggests that this magnitude of affinity difference is sufficient for distinction between a single $d^{6m}\text{A}$ in a DNA sequence and DNA sequences without any $d^{6m}\text{A}$. Thus, the 7C7:C5 MAb is ideal for uses in diagnostics.

One example for the use of the 7C7:C5 MAb would be through its application to a relatively fast and simple diagnostic format such as an ELISA or a lateral flow
chromatography device. For instance, a TaqI MT (M.TaqI) site (TCG^{6m}A) happens to be present in bovine viral diarrhea virus type II (BVDV-2) but not in BVDV-1. Infection with BVDV-2 is much more pathogenic to cattle than BVDV-1, in which BVDV-2 can lead to hemorrhagic diarrhea. This rapid diagnostic strategy can even be applied to two different strains of influenza A virus that infect humans: (A/Michigan/1/1985(H3N2)) #AF008872, a strain of unspecified pathogenesis, and (A/New_York/1/18(H1N1)) #AF116576, a strain that resulted in a rapid and acute pathogenesis of an infected 30 year old male ultimately resulting in death^{175}. While the hemagglutinin (HA) genes of both of these strains contain a M.TaqI site, only the aforementioned H1N1 HA gene contains an M.EcoRI (GA^{6m}ATTC) site. As a result, upon isolating the HA gene and methylating with an M.EcoRI, immunochemical detection of the M.EcoRI site with the 7C7:C5 MAb can be used to distinguish between these two strains of influenza A.

Whether or not the use of \( ^{6m}A_{os} \) or \( ^{6m}A \) makes a difference in the binding affinity is a rather important question to its potential future use as a therapeutic delivery tool for small interfering RNA (siRNA). The difference between \( ^{6m}A_{os} \) and \( ^{6m}A \) is merely an additional alcohol group that occurs in ribose but not deoxyribose. The 5’ oxygen of \( ^{6m}A \) was shown to be involved in a H-bond with Tyr L48 in the flexible Fv docking model of the 7C7:C5 Fv. As a result, if a difference in binding affinity did exist, the most likely difference in binding affinity would occur through an additional H-bond in the favor of the ribonucleoside \( \left(^{6m}A_{os}\right) \). This additional interaction would favor the use of the 7C7:C5 Fv towards binding of \( ^{6m}A_{os} \) in RNA as opposed to \( ^{6m}A \) in DNA.

Nevertheless, the general methods of immunization described by Erlanger and Beiser^{98} that provided the background information used for the immunization process in
production of the 7C7:C5 MAb decisively stated that the production of anti-DNA antibodies was successful by these immunization methods but for some unknown reason attempts at producing anti-RNA antibodies did not show the same success. This occurrence may be due to the inherent requirement of high specificity involved in RNA binding. High affinity binding of RNA has been shown to predominantly involve hydrophobic stacking interactions and hydrogen bonding, but also specific charged interactions in key positions \(^83, 176-179\). Furthermore, binding of A-RNA helices involves a deeper and narrower groove than the major groove of B-DNA making it more difficult for proteins to fit into these grooves \(^179\). Fortunately, RNA is somewhat more plastic than DNA and leaves much of its surface area for interaction at the ends of its grooves, in which unstacked bases in the RNA strand can be either pulled into binding pockets or positioned accordingly to allow the protein for sequence-specific binding. As a result, the 7C7:C5 Fv may be able to bind to a \(^{6m}\text{A}_{os}\) nucleotide in RNA if it is in a more accessible position along the RNA strand.

An Fv has numerous advantages in terms of therapeutics over a complete MAb. Single-chain (sc) Fvs retain high affinity for their target antigens, do not exhibit undesirable recognition of non-specific cells associated with crystallizable constant fragments (Fc\(s\)), and have far shorter half-lives than complete MAbs limiting the possibility of eliciting an undesirable immune response \(^{180-182}\). Also, Fvs can be used to construct recombinants via an expression vector. Recombinant technology can be used to humanize an antibody or to produce a fusion protein.

A fusion protein composed of protamine for the binding of siRNAs covalently linked to the C terminus of an anti-glycoprotein (gp) 160 Fab, a HIV-1 envelope
glycoprotein, was used in a dose dependent manner for successful delivery of siRNAs to

gp160 or cells transfected with gp160\textsuperscript{182}. This treatment resulted in a 49% decrease of

HIV-1 productively infected cells. The same fusion protein was also used for siRNA
delivery for tumor suppression of murine melanoma B16 cells, in which a maximum

suppression of three-fold was obtained. This example of siRNA delivery could easily be

applied to any target using bispecific dimeric Fvs (diabodies) or even bispecific Fv-CH3-

Fv constructs (minibodies) in which one Fv could be used to carry the siRNA and the

other Fv could be used to target entry or gateway receptors on cancer cells or viral

infected cells.
REFERENCES


96. Hoffman, J.F. English translation: *Production of a Monoclonal Antibody that Specifically Recognizes \(^6m\text{Adenine})*. O.J. López Laboratory in Argentina (2002).


APPENDIX A

List of Online Resources

AutoDock, http://autodock.scripps.edu/

Cygwin, http://cygwin.com/


Fermentas, http://www.fermentas.com/


Invitrogen, http://www.invitrogen.com/content.cfm?pageid=1

Modeller, http://salilab.org/modeller/


RCSB Protein Data Bank, http://www.rcsb.org/pdb/home/home.do


The University of Michigan DNA Sequencing Core, http://seqcore.brcf.med.umich.edu/

UCSF Chimera, http://www.cgl.ucsf.edu/chimera/

APPENDIX B

Modeller9v4 Command Files

# Homology modelling by the automodel class
from modeller import *  # Load standard Modeller classes
from modeller.automodel import *  # Load the automodel class

log.verbose()    # request verbose output
env = environ()  # create a new MODELLER environment to build this model in

# directories for input atom files
env.io.atom_files_directory = ['.', '../atom_files']

a = automodel(env,
    alnfile = '7C7C5Fv.ali',     # alignment filename
    knowns = ('1QOK','1HQ4','1F8T','1S5I','32C2','1AY1','1KCU','3CFE','2A0L','1ORS','1EZV','1BAF','1KEN','1KCS','2FON','1I9I','1E6O','2FAT','1AIF'),
    sequence = '7C5U')  # code of the target

a.starting_model= 1     # index of the first model
a.ending_model  = 1     # index of the last model
# (determines how many models to calculate)
a.make()  # do the actual homology modelling

Figure 38. Modeller9v4 command file for homology modeling of the antigen unbound template model of the 7C7:C5 Fv.
# Homology modelling by the automodel class
from modeller import *              # Load standard Modeller classes
from modeller.automodel import *    # Load the automodel class

log.verbose()    # request verbose output
env = environ()  # create a new MODELLER environment to build this model in

# directories for input atom files
env.io.atom_files_directory = ['.', '../atom_files']

a = automodel(env,
              alnfile = '7C7C5Fvb.ali',     # alig nment filename
              knowns   = ('1QOK','1ORS','1CP8','1F9O','1BAF','1KCR','1KEN','2A0L','1BGX','1KC5',
                          '1NCW','3CFD','1EZV','1S5I','1KCS','2FON','1I9I','1E6J','2FD6',
                          '2FBJ'),     # codes of the templates
              sequence = '7C5V')             # code of the target

a.starting_model= 1  # index of the first model
a.ending_model  = 1   # index of the last model
a.make()                            # do the actual homology modelling

Figure 39. Modeller9v4 command file for homology modeling of the antigen bound template model of the 7C7:C5 Fv
APPENDIX C

AutoDock-4.0 Rigid Fv Docking Log Files

MODEL        5
USER    Run = 5
USER    Cluster Rank = 1
USER    Number of conformations in this cluster = 1
USER    RMSD from reference structure       = 32.352 A
USER
USER    Estimated Free Energy of Binding    =   -5.15 kcal/mol
[= (1)+(2)+(3)-(4)]
USER
USER    Estimated Dissociation Constant, K_d = 168.88 uM
(micromolar)           [Temperature = 298.15 K]
USER
USER    (1) Final Intermolecular Energy     =   -5.50 kcal/mol
USER    vDW + Hbond + desolv Energy        =   -5.47 kcal/mol
USER    Electrostatic Energy               =   -0.03 kcal/mol
USER    (2) Final Total Internal Energy     =   -0.80 kcal/mol
USER    (3) Torsional Free Energy          =   +1.10 kcal/mol
USER    (4) Unbound System's Energy        =   -0.06 kcal/mol

Figure 40. AutoDock-4.0 docking log file for the most favorable binding mode of d\textsuperscript{6mA} to the 7C7:C5 Fv using rigid Fv docking.
Figure 41. AutoDock-4.0 docking log file for the most favorable binding mode of dA to the 7C7:C5 Fv using rigid Fv docking.
Figure 42. AutoDock-4.0 docking log file for the second most favorable binding mode of A-6m A-T to the 7C7:C5 Fv using rigid Fv docking.
APPENDIX D

AutoDock-4.0 Docking Log Files for Fv with Five Flexible Residues

MODEL       19
USER    Run = 19
USER    Cluster Rank = 1
USER    Number of conformations in this cluster = 1
USER    RMSD from reference structure       = 1.802 Å
USER    Estimated Free Energy of Binding    =  -14.57 kcal/mol
USER    Estimated Dissociation Constant, $K_d$ = 21.04 pM
USER    (picomolar)  
USER    [Temperature = 298.15 K]
USER    (1) Final Intermolecular Energy     =   -5.44 kcal/mol
USER    vDW + Hbond + desolv Energy     =   -3.51 kcal/mol
USER    Electrostatic Energy            =   -0.05 kcal/mol
USER    Moving Ligand-Fixed Receptor    =   -3.56 kcal/mol
USER    Moving Ligand-Moving Receptor    =   -1.88 kcal/mol
USER    (2) Final Total Internal Energy     =  -11.45 kcal/mol
USER    Internal Energy Ligand          =   -0.67 kcal/mol
USER    Internal Energy Receptor        =  -10.78 kcal/mol
USER    (3) Torsional Free Energy      =   +1.10 kcal/mol
USER    (4) Unbound System's Energy        =   -1.23 kcal/mol

Figure 43. AutoDock-4.0 docking log file for the most favorable binding mode of d$_{6m}$A to the 7C7:C5 Fv using flexible Fv docking.
MODEL  23
USER  Run = 23
USER  Cluster Rank = 16
USER  Number of conformations in this cluster = 1
USER  RMSD from reference structure = 3.839 Å
USER  Estimated Free Energy of Binding = -13.45 kcal/mol
[= (1)+(2)+(3)-(4)]
USER  Estimated Dissociation Constant, $K_d$ = 138.84 pM
    (picomolar) [Temperature = 298.15 K]
USER
USER  (1) Final Intermolecular Energy = -3.59 kcal/mol
USER  vdw + Hbond + desolv Energy = -2.16 kcal/mol
USER  Electrostatic Energy = -0.06 kcal/mol
USER  Moving Ligand-Fixed Receptor = -2.22 kcal/mol
USER  Moving Ligand-Moving Receptor = -1.37 kcal/mol
USER  (2) Final Total Internal Energy = -11.55 kcal/mol
USER  Internal Energy Ligand = -0.72 kcal/mol
USER  Internal Energy Receptor = -10.83 kcal/mol
USER  (3) Torsional Free Energy = +1.10 kcal/mol
USER  (4) Unbound System's Energy = -0.59 kcal/mol

Figure 44. AutoDock-4.0 docking log file for the sixteenth most favorable binding mode of dA to the 7C7:C5 Fv using flexible Fv docking. This binding mode was selected for the docking of dA to the 7C7:C5 Fv due to its similarity to the most favorable binding modes in the docking of d$^6$mA and the d$^6$mA moiety of A$^-6$mA-T to the 7C7:C5 Fv.
MODEL       13
USER    Run = 13
USER    Cluster Rank = 3
USER    Number of conformations in this cluster = 3
USER    RMSD from reference structure = 3.360 Å
USER    Estimated Free Energy of Binding = -14.22 kcal/mol
USER    Estimated Dissociation Constant, $K_d$ = 37.57 pM
(USER    (1) Final Intermolecular Energy = -4.82 kcal/mol
(USER    vDW + Hbond + desolv Energy = -2.74 kcal/mol
(USER    Electrostatic Energy = -0.01 kcal/mol
(USER    Moving Ligand-Fixed Receptor = -2.75 kcal/mol
(USER    Moving Ligand-Moving Receptor = -2.08 kcal/mol
(USER    (2) Final Total Internal Energy = -11.09 kcal/mol
(USER    Internal Energy Ligand = -0.71 kcal/mol
(USER    Internal Energy Receptor = -10.38 kcal/mol
(USER    (3) Torsional Free Energy = +1.10 kcal/mol
(USER    (4) Unbound System's Energy = -0.59 kcal/mol

Figure 45. AutoDock-4.0 docking log file for the third most favorable binding mode of dA to the 7C7:C5 Fv using flexible Fv docking. This binding mode shows dA docked deep in the binding pocket of the 7C7:C5 Fv, but the dA is docked in an orientation that is horizontally opposite of the most favorable binding mode found for d$^6$mA.
MODEL 31
USER Run = 31
USER Cluster Rank = 1
USER Number of conformations in this cluster = 1
USER
USER RMSD from reference structure = 9.702 Å
USER
USER Estimated Free Energy of Binding = -14.36 kcal/mol
[= (1)+(2)+(3)-(4)]
USER
USER Estimated Dissociation Constant, $K_d$ = 29.63 pM
(picomolar) [Temperature = 298.15 K]
USER
USER (1) Final Intermolecular Energy = -7.92 kcal/mol
USER vdw + Hbond + desolv Energy = -5.61 kcal/mol
USER Electrostatic Energy = -0.14 kcal/mol
USER Moving Ligand-Fixed Receptor = -5.75 kcal/mol
USER Moving Ligand-Moving Receptor = -2.18 kcal/mol
USER (2) Final Total Internal Energy = -11.45 kcal/mol
USER Internal Energy Ligand = -1.35 kcal/mol
USER Internal Energy Receptor = -10.11 kcal/mol
USER (3) Torsional Free Energy = +4.12 kcal/mol
USER (4) Unbound System's Energy = -0.89 kcal/mol

Figure 46. AutoDock-4.0 docking log file for the most favorable binding mode of A-\textsuperscript{6m}A-T to the 7C7:C5 Fv using flexible Fv docking.