

NMR RESONANCE ASSIGNMENT OF THE THERAPEUTIC RNA APTAMER

MACUGEN IN COMPLEX WITH ITS *IN VIVO* TARGET, THE HEPARIN

BINDING DOMAIN OF VEGF₁₆₅

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**NMR Resonance Assignment of the Therapeutic RNA Aptamer Macugen in
Complex with its *in Vivo* Target, the Heparin Binding Domain of VEGF₁₆₅**

Thesis directed by Professor Arthur Pardi

Aptamers consist of nucleic acids that are selected from an *in vitro* library in order to bind a specific target or catalyze a reaction. Many aptamer-ligand structures have been solved in recent years to characterize the molecular mechanisms that explain the high affinity and specificity observed in these interactions. To this end, NMR resonance assignment experiments were performed as a necessary first step to calculating the solution structure of a therapeutically-active RNA aptamer, Macugen, bound to its *in vivo* target, the heparin binding domain (HBD) of vascular endothelial growth factor 165 (VEGF₁₆₅). Though the resonance assignment of Macugen is challenging due to its inability to be isotopically-labeled, techniques were employed that take advantage of the unique chemical modifications of this nucleic acid that are not present in traditional, unmodified RNA. Using the information obtained from two-dimensional heteronuclear (¹⁹F, ¹H) and homonuclear (¹H, ¹H) experiments, assignments were made for a sizable number of Macugen atoms. The assignments made in this work will be necessary to calculate a solution structure to better understand the molecular mechanisms that give rise to the high affinity and specificity observed in the Macugen-HBD complex.

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CHAPTER 1

Background and Introduction

1.1 Introduction to aptamers and the SELEX process

Aptamers consist of RNA or DNA molecules that are produced *in vitro* with the purpose of binding a specific target or catalyzing a certain reaction [1-3]. For instance, there currently exist aptamers that bind a variety of biomolecules such as proteins, viruses, nucleic acids, aminoglycosides, vitamins, cofactors, and even whole tissues via cell-surface protein recognition [1, 4-6]. Additionally, aptamers have also been developed to bind an assortment of synthetic molecules, including drugs and organic dyes [7-9]. Like antibodies, aptamers have been shown to bind targets with remarkable affinity and specificity [7, 9, 10]. Importantly, many aptamers have also been produced to catalyze specific chemical reactions, such as the hydrolysis and isomerization of small molecules [3, 11]. Thus, aptamers have potential for use in a wide array of applications, including biosensing, therapeutics, bioanalytical chemistry, and tools for the biotechnology industry [12-15].

The process by which aptamers are produced *in vitro* is known as Systematic Evolution of Ligands by EXponential Enrichment, or SELEX. Functional RNA or DNA molecules are obtained by first constructing large libraries of unique nucleic acids (up to 10^{14}) containing regions of random sequence, and by then applying selective pressures to isolate species within the library that are capable of binding a ligand [1, 2]. For example, a large pool of randomized nucleic acids may be incubated with a target protein ligand and then subjected to partitioning procedures

that sequester sequences that exhibit binding affinity to the protein [9]. The isolated species are subsequently amplified and subjected to further rounds of selection, resulting in an enriched fraction of nucleic acids from the original pool that are able to bind a specific target with considerable affinity. If desired, the SELEX experiment can be designed to produce species capable of catalyzing specific reactions (rather than solely binding a target) [16].

The basis for the discrimination and affinity that aptamers display for ligands has been a topic of great interest [17-19]. For example, considerable work has been done to understand the binding mechanisms involved in complex formation. It has been shown that many aptamers do not adopt rigid conformations prior to ligand binding [20]. Rather, these complexes appear to form via induced-fit mechanisms, or so-called conformational capture [19-22]. Importantly, this adaptive recognition process closely parallels the mechanism by which many natural RNA molecules (and enzymes) bind *in vivo* targets with high affinity and specificity, although it has been proposed that aptamers selected purely for high affinity don't necessarily carry the ability to completely discriminate between very closely-related targets [17, 18, 20, 21]. Utilizing high affinity *and* specificity as selective pressures in SELEX experiments may be necessary to achieve high affinity/specificity for some aptamer-ligand systems [18].

Also of interest are the types of molecular interactions that give rise to the high affinity and specificity observed in aptamer-ligand complexes. Accordingly, a variety of aptamer-ligand structures have been solved in order to elucidate these interactions [4, 6, 22-26]. Features common to aptamer-ligand complexes include

shape/charge complementarity between aptamer/ligand binding surfaces, specific hydrogen bonding networks, and unique aromatic stacking [8, 19]. It has also been found that aptamers tend to fold around and enclose large areas of the ligand, thereby allowing a significant number of specific intramolecular contacts to be made [19]. Interestingly, several novel RNA//DNA folds have been discovered by studying aptamer-ligand complexes [24, 27]. The emergence of additional aptamer-ligand structures continues to promote understanding of the molecular mechanisms by which these nucleic acids bind to such diverse ligands with high specificity and affinity. Indeed, it is a long-term goal of this project to understand the molecular basis for one such complex by examining its structure.

1.2 Vascular Endothelial Growth Factor (VEGF) as a SELEX target

Vascular endothelial growth factor (VEGF) is a hypoxia-inducible angiogenic growth factor which plays a vital role in many physiological processes [28]. Cells deficient in oxygen express hypoxia-inducible factor (HIF), a transcription factor, which stimulates the production and extracellular release of VEGF [29]. VEGF, a 45 kDa homodimer, binds to its receptors on the surface of vascular endothelial cells, leading to phosphorylation of the receptors and a subsequent signaling pathway that promotes nascent blood vessel growth [28, 30]. VEGF is a vital component of many physiologically-important processes, including regular maintenance of the vasculature, embryogenesis, and wound healing [31, 32]. However, in addition to its normal functions, VEGF has also been widely implicated in a number of pathological

processes including inflammation, rheumatoid arthritis, cancer, and the wet form of age-related macular degeneration (AMD) [33, 34].

In humans, four predominant VEGF splice-variants have been identified and are designated according to their number of amino acids: VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆ [28]. All variants contain an identical N-terminal receptor binding domain (RBD), though they are otherwise unique in their structural make-up and functionality [28, 31]. VEGF₁₈₉ and VEGF₂₀₆ are almost exclusively segregated to the extracellular matrix, whereas VEGF₁₂₁ and VEGF₁₆₅ are soluble and can diffuse once exported. In addition, VEGF₁₆₅ contains a unique 55-amino acid heparin-binding domain (HBD) found at the C-terminus that is not present in the other soluble variant, VEGF₁₂₁ [35, 36]. Notably, VEGF₁₆₅ has been identified as the primary isoform implicated in disease [28].

Due to its prominent role in pathological conditions, VEGF has been identified as a significant therapeutic target [37, 38]. One historical challenge to designing VEGF inhibitors is that it is difficult to specifically target the disease-implicated isoform (VEGF₁₆₅) [39]. As stated previously, all VEGF variants contain a receptor binding domain (RBD), and one of the first inhibitors developed against VEGF was the soluble form of the VEGF receptor [40]. While limiting or possibly halting the progression of disease, it is unknown whether species that therapeutically target VEGF via the RBD may also negatively affect blood vessel formation during normal, healthy processes since all isoforms contain this moiety [39]. Several successful and clinically-effective agents have been produced that target all VEGF variants indiscriminately, although development of inhibitors that bind only VEGF₁₆₅

remains desirable [39, 41]. Importantly, because SELEX can produce species capable of binding a ligand with high affinity *and* specificity, this method was viewed as a fitting candidate for developing therapeutic agents that can effectively bind and discriminate between various isoforms of a protein such as VEGF.

To this end, the SELEX process was employed by Jellinek and coworkers in the mid-nineties to develop nucleic acid aptamers that bind the 165-amino acid VEGF isoform [42]. The aptamers that were obtained from this original SELEX experiment were enriched from a library of 10^{14} unique RNA molecules, each containing a region of 30 randomized nucleotides (the evolved region). Aptamers were obtained that bound VEGF₁₆₅ with high affinities ranging from 0.2 nM to 20 nM, and can be categorized into six main families based on consensus in sequence in the randomized region. The aptamers from this experiment are shown in **Figure 1.1**.

Importantly, it was found that heparin effectively competes with these aptamers for VEGF₁₆₅ binding, suggesting that these nucleic acids may interact with the heparin binding domain (HBD) of the protein (a unique component found in VEGF₁₆₅ that is not present in the other soluble isoform, VEGF₁₂₁) [28, 42]. Therefore, it is likely that these aptamers possess the ability to discriminate between the soluble isoforms of VEGF—a beneficial property that other inhibitors to the growth factor did not previously exhibit at the time [39, 41]. In addition, the enriched nucleic acids were shown to effectively inhibit binding of VEGF₁₆₅ to its receptors on human umbilical vein endothelial cells in a concentration-dependent manner [42]. Hence, this work provided robust evidence that the SELEX process could potentially be employed to produce therapeutically-active species that target the disease-

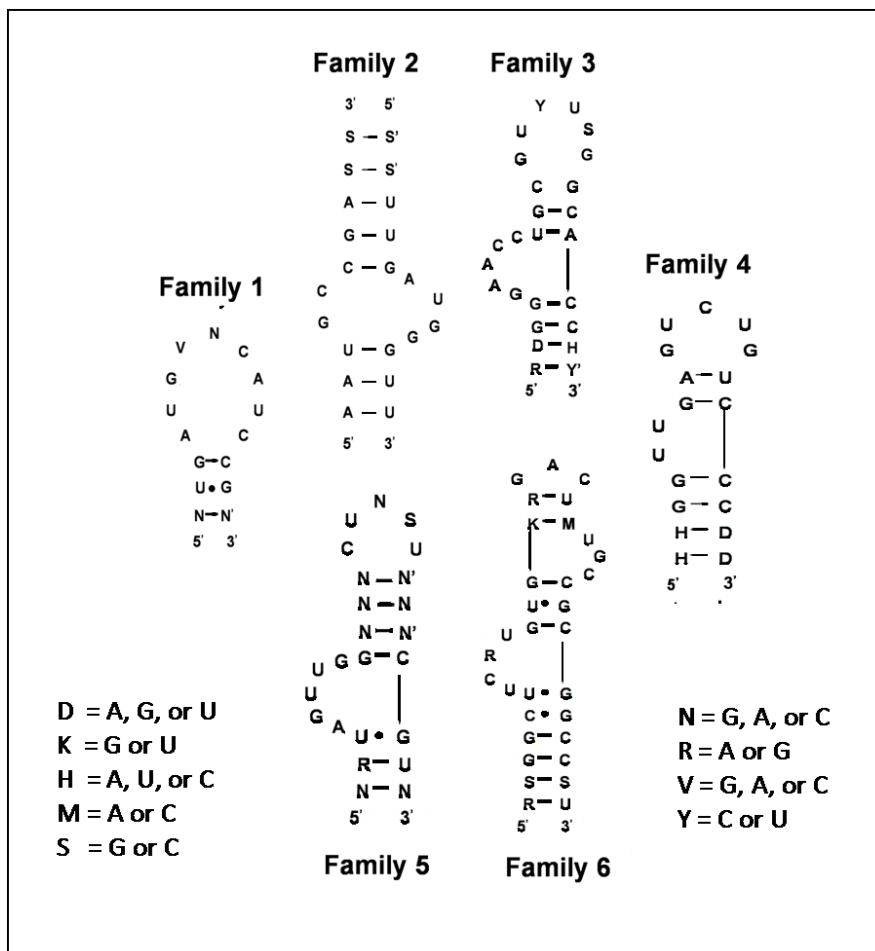


Figure 1.1 Unmodified RNA aptamers selected against VEGF₁₆₅. Jellinek *et al.*

selected aptamers from a library of 10^{14} unique RNA molecules, each with a region of 30 random nucleotides. Aptamers found to effectively bind VEGF₁₆₅ can be divided into six main families based on sequence consensus.

implicated form of VEGF with high affinity *and* specificity. Aptamers were truncated to their minimally-functional sequences and, to further enhance resistance to degradation, most purine residues were changed to 2'-OMe-containing analogs. After testing for retention of binding activity, the resulting product was a series of degradation-resistant, chemically-modified RNA aptamers that bind VEGF₁₆₅ with extremely low dissociation constants ranging from 49 pM to 130 nM [42].

1.3 Development of chemically-modified RNA aptamers to bind VEGF₁₆₅

Chemical and physical stability are important determinants of pharmaceutical efficacy [43]. For example, RNA aptamers used in therapeutic applications must be resistant to base-catalyzed hydrolysis. Various strategies are currently used to stabilize aptamers, including substitution of the 2'-OH on ribose sugars with 2'-fluoro (2'-F) or 2'-O-methyl (2'-OMe) groups in order to prevent the 2' oxygen from attacking the phosphate backbone [13]. Due to the possible therapeutic potential of previous aptamers developed to target VEGF₁₆₅, Ruckman and coworkers carried out three separate SELEX experiments with 2'-F pyrimidine nucleotides to obtain a set of degradation-resistant aptamers that bind VEGF₁₆₅ with high affinity [44].

The chemically-modified (2'-F pyrimidine and 2'-OMe purine) RNA aptamers produced from this work can be grouped into three distinct families based on consensus sequence in the random sequence region (30 and 40 nucleotides). These aptamers do not show binding affinity for VEGF₁₂₁, indicating they are able to effectively discriminate between the two soluble forms of VEGF. Additionally, similar to the original unmodified RNA aptamers, it is likely that the HBD of

VEGF₁₆₅ is heavily involved in the aptamer-protein binding interaction; experiments showed photo-crosslinking between uracil residues of the aptamers and Cys¹³⁷ located in the HBD of the protein [42]. Finally, it was shown that these aptamers were able to inhibit binding of VEGF₁₆₅ to its receptor on porcine aortic endothelial cells, thereby demonstrating their possible use as potent inhibitors [42]. Due to their resistance to degradation, their ability to bind to the disease-implicated isoform of VEGF with high affinity/specificity, and their ability to inhibit binding of VEGF₁₆₅ to its receptors, these aptamers were considered to have high therapeutic potential.

1.4 Characteristics of the Macugen-VEGF₁₆₅ complex and the role of the HBD in binding

One of the chemically-modified RNA aptamers that targets VEGF₁₆₅ was approved by the FDA in 2004 to treat the wet form of age-related macular degeneration (AMD), thus becoming the first therapeutic aptamer on the market [37]. Known as Macugen (pegaptanib sodium), this 28-nucleotide aptamer is delivered via intraocular injection and binds VEGF₁₆₅ with very high affinity ($K_d = 50$ pM) to limit progression of the eye disease. Additionally, Macugen significantly decreases vascular permeability (the ability of small molecules and ions to penetrate the vascular wall)—a pathological characteristic of wet AMD [44, 45]. The aptamer is highly resistant to degradation; chemical modifications include 2'-F pyrimidines, 2'-OMe groups on all but two purines, and an added thymidine to the 3' terminus via a reversed 3'-3' linkage. The sequence and secondary structure of Macugen are shown in **Figure 1.2**.

Work has been done to show that Macugen specifically targets the 55-amino acid HBD of VEGF₁₆₅—an N-terminal domain that is not present in VEGF₁₂₁, the other soluble form of the growth factor [45]. The ability of Macugen to bind the HBD likely gives rise to its ability to discriminate between the two soluble isoforms to directly target the disease-implicated variant. As mentioned previously, Macugen demonstrated photo-crosslinking between 5-iodo-modified U14 in the hairpin loop and Cys¹³⁷ of the HBD [44]. Additionally, *in vivo* experiments have shown that free HBD can efficiently compete with the full-length protein for aptamer binding, thus providing further evidence that this domain is targeted by Macugen [45]. Furthermore, studies have determined that the majority of the binding energy comes from interactions between Macugen and the HBD; the aptamer binds free HBD with a dissociation constant of 12 nM ($\Delta G^\circ = -10.8$ kcal/mol), compared to a K_d of 50 pM ($\Delta G^\circ = -14.0$ kcal/mol) for the full-length protein [45].

Nuclear magnetic resonance (NMR) methods have also been employed to confirm that the HBD is primarily targeted in the Macugen-VEGF₁₆₅ interaction. Chemical shifts of protein backbone amides can be monitored via ¹⁵N-¹H correlation experiments (¹⁵N, ¹H HSQC) to observe different folding patterns or changes in the local chemical environment—common occurrences in complex formation [46, 47]. ¹⁵N, ¹H HSQC experiments have shown that the chemical shifts of many backbone amide residues in the HBD are significantly perturbed upon binding to Macugen, indicating that there are direct interactions between the HBD and aptamer [45].

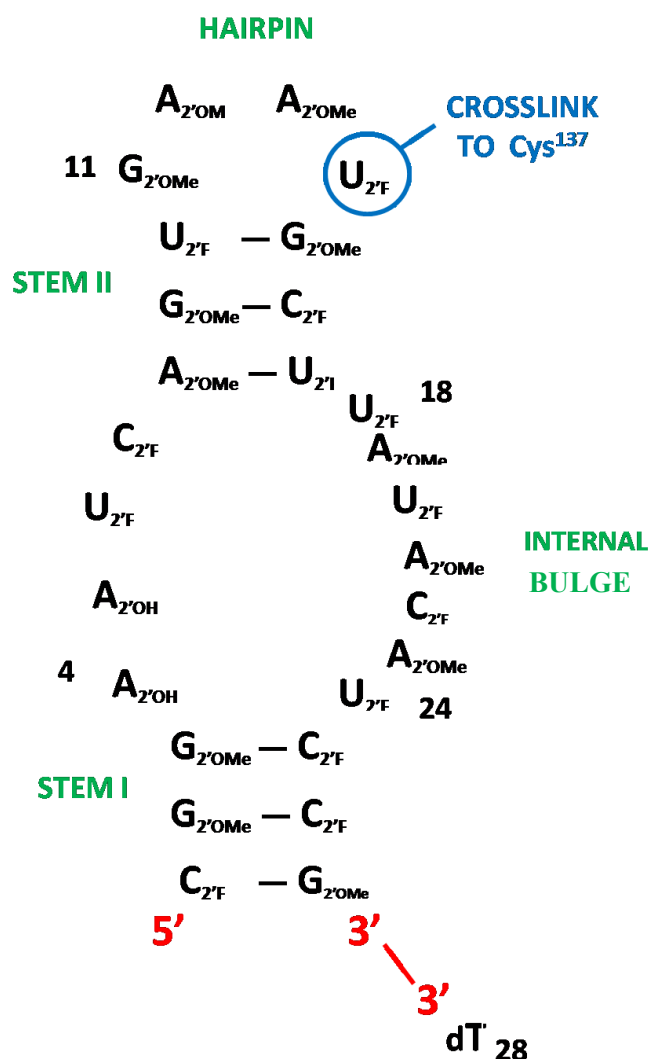


Figure 1.2 The sequence, secondary structure, and chemical modifications of Macugen. This 28-nt, chemically-modified RNA was obtained through SELEX and was found to effectively inhibit angiogenesis by targeting the heparin binding domain (HBD) of VEGF₁₆₅. Except for A4 and A5, all purines contain 2'-O-methyl (2'-OMe) modifications for increased stability and nuclease resistance. All pyrimidines contain a 2'-fluoro (2'-F) moiety. U14 was found to form a crosslink with Cys¹³⁷ of VEGF₁₆₅. The 3' terminus of the aptamer contains a deoxythymidine (dT₂₈) reverse-linked (3'—3') to G₂₇ to protect against exonuclease activity.

Equally important, the unique ^{15}N , ^1H HSQC fingerprint of free HBD bound to Macugen is also seen in the ^{15}N , ^1H HSQC spectrum of the full-length protein bound to the aptamer, demonstrating that the HBD adopts the same conformation in both the Macugen-HBD and Macugen-VEGF₁₆₅ complexes [45].

NMR studies have also shown that Macugen adopts the same conformation when bound to either full-length VEGF₁₆₅ or the HBD alone [45]. 1D imino proton spectra of the HBD- and full-length-bound complexes are extremely similar, suggesting that the nucleic acid's secondary structure is equivalent in the complexes [45]. Additionally, ^{31}P NMR can be used as a sensitive probe for detecting even slight conformational differences in ribose-phosphate backbones of nucleic acids [48]. 1D ^{31}P NMR spectra of Macugen complexed with both the HBD and the full-length VEGF₁₆₅ are essentially identical, providing further evidence that Macugen conformation is the same in these complexes [45]. Notably, Ca^{2+} is a required component for Macugen-VEGF₁₆₅ binding, and this dependence has also been observed for Macugen-HBD complex formation [44, 45]. However, the presence of Ca^{2+} does not affect NMR spectra of either free species (Macugen or HBD), suggesting that this divalent cation is mediating an interaction between the aptamer and HBD [45]. The above work provides solid evidence that the HBD is specifically targeted by Macugen, and that this domain can be used for further studies in order to understand the binding mechanisms/interactions of the aptamer with its protein ligand.

1.5 The need for Macugen NMR resonance assignments

The remarkably high affinity and specificity exhibited by Macugen for VEGF₁₆₅ has generated interest in understanding the fundamental mechanisms that govern this interaction. To examine the types of molecular interactions occurring in the Macugen-HBD complex, NMR is being used to obtain a solution structure. Solution structures are solved using dihedral angle constraints and inter-atomic distances derived from nuclear Overhauser effects (NOEs) to calculate conformations with low free energies [47]. To do this, it is necessary to first assign resonances in NMR spectra to their associated nuclei. A historic difficulty with assigning resonances of large biomolecules or complexes is that, due to the large number of atoms and associated NMR signals, it is often challenging to make unambiguous assignments [49]. Spectral overlap and crowding of peaks in conventional homonuclear 1D and 2D experiments become highly problematic as the size of the biomolecule increases. Modern methods have addressed this by making use of isotopically-labeled (¹⁵N, ¹³C, ²H) biomolecules and unique multidimensional heteronuclear NMR experiments [47]. One advantage of isotopic labeling is that peaks can be separated in multiple dimensions, thereby reducing spectral overlap/crowding and increasing the odds of unambiguous resonance assignment.

Expression of isotopically-labeled proteins has become a common practice, and the use of this strategy in conjunction with multi-dimensional heteronuclear NMR has been employed in our lab to confidently assign the resonances of most atoms of the HBD in complex with Macugen [unpublished data]. The process is fairly straight-forward; multidimensional heteronuclear NMR experiments are first

conducted that correlate resonances belonging to atoms of individual spin systems (amino acids), and then the spin systems are assembled sequentially using knowledge of the protein's sequence and experiments that correlate resonances across the amide bond [47]. Thus, once the spin systems are connected sequentially, chemical shifts of atoms belonging to each amino acid can be confidently assigned. A similar process is typically used to accurately assign chemical shifts in isotopically-labeled nucleic acids. Experiments are done that reveal resonances in individual spin systems (sugars and bases), and then the spin systems are connected sequentially (sugar-to-base walking) using prior knowledge of the nucleic acid sequence and experimentally-obtained NOEs between atoms on neighboring residues.

Unfortunately, Macugen cannot be isotopically labeled, thereby making resonance assignment of its atoms a challenging task due to reasons described earlier. First, *in vitro* transcription of Macugen using 2'-F pyrimidines is not efficient enough to yield quantities of the aptamer needed for multi-dimensional NMR experiments. Also, it has been shown that binding is not retained when Macugen is transcribed using 2'-OH pyrimidines [unpublished data]. For these reasons, Macugen needs to be chemically-synthesized in order to produce the required quantities for NMR. At present, isotopically-labeled 2'-F phosphoramidites are not available for use in synthesis, so isotopically-labeled Macugen cannot be obtained for use in NMR resonance assignment experiments. In order to assign resonances of Macugen atoms, more traditional NMR experiments must be employed, and nonstandard strategies must be developed to assign chemical shifts with a high degree of certainty.

1.6 Project description and overview of thesis

The long-term goal of this project is to understand the types of molecular interactions that allow Macugen to specifically target the HBD of VEGF₁₆₅ with extremely high affinity and specificity. To this end, work is being done to obtain a solution structure of the Macugen-HBD complex. This thesis specifically addresses the need for NMR resonance assignments of the bound aptamer with the goal of obtaining a structure in the future. Assignments used in structure calculation must be accurate, so a novel approach was devised that used the 2'-F and 2'-OMe chemical modifications to strategically identify resonances. The modifications allow for more confident assignment of Macugen compared to unmodified RNA of similar size.

CHAPTER 2

Methods

2.1 Preparation of HBD of VEGF₁₆₅ for NMR studies

The ¹⁵N-labeled HBD of VEGF₁₆₅ was expressed and purified for NMR experiments in complex with Macugen as described previously [45]. Protein was expressed in *P. Pastoris* for a period of 48 hours (slightly differing from the original protocol which calls for an expression time of 36 hours). Though the ¹⁵N- labeling of this protein is not necessary for sequential assignment of Macugen, it does allow for future multidimensional NMR experiments that probe the behavior of the HBD in the complex. The HBD was purified on a heparin Sepharose column and exchanged into the following buffer (NMR buffer): 100% D₂O, 100 mM NaCl, 10 mM Tris-d11 (pH=7.0), 1 mM CaCl₂, 0.01 mM EDTA, and 0.05% NaN₃. The protein was concentrated using Centricon YM-3 centrifugal filter devices to a volume of 310 μL, and the concentration was found to be 1 mM from a BCA assay.

2.2 Preparation of the Macugen-HBD complex for NMR studies

Non-pegylated Macugen was obtained from NeXstar pharmaceuticals. The amount of aptamer necessary for producing a 0.95 mM NMR sample (at a volume of 310 μL) was aliquoted and buffer-exchanged into D₂O (pH=7.0). After buffer exchange, the aptamer was lyophilized and subsequently dissolved into the HBD sample to form the complex. It is important to note that NMR studies conducted to sequentially assign Macugen should optimally be performed in a slight excess of

HBD (as opposed to a 1:1 ratio). This excess helps to ensure that all Macugen is in complex with HBD, and that signals obtained in NMR studies can confidently be attributed to the bound form. The concentration of HBD in the experiments was 1 mM, thus Macugen was chosen to be at a concentration of 0.95 mM. To ensure that all aptamer was bound, a 2D ^1H , ^1H TOCSY spectrum of this complex was obtained and then compared to spectra previously acquired for free Macugen. No H5/H6 peaks arising from the free aptamer were seen in the prepared complex meaning all aptamer was bound. Additionally, the TOCSY spectrum was compared to that of a previously-prepared Macugen-HBD complex, and they were found to be identical. Therefore, it was determined that the NMR sample was sufficient for assignment studies.

2.3 Acquisition of a 2D ^1H , ^1H TOCSY spectrum to identify pyrimidine H5/H6 resonances in the Macugen-HBD complex

A ^1H , ^1H TOCSY spectrum was first collected to obtain H5/H6 resonances of Macugen 2'-F pyrimidines [50]. The experiment was performed at 25°C on a 500 MHz Varian spectrometer using a $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ triple resonance probe with z-axis pulsed field gradients. The sequence was run with a 48 ms mixing time (achieved by using 24 MLEV-17 cycles with a 30 μs spinlock time), and a 32-step z-filter phase cycle was applied to remove zero quantum peaks [27]. 2048 complex points were taken in t_2 , and 195 complex points in t_1 . The sweep-width was set to 5006 Hz in both dimensions. The data was processed using NMRPipe, and cosine bell window functions were applied in each dimension. Cross-peaks corresponding to pyrimidine

H5/H6 resonances were located and temporarily given arbitrary designations until they were assigned to specific pyrimidines.

2.4 Acquisition of a 2D ^{19}F , ^1H HMQC to identify pyrimidine H1', H2', H3' and 2'-F resonances in the Macugen-HBD complex

A unique property of Macugen that makes assignment easier compared to unlabeled RNA of similar size is its chemical modifications. Fluorine is an NMR-active nucleus, so experiments can be employed that use this nucleus to aid in resonance assignment. One way in which this group can be exploited is to perform through-bond heteronuclear correlation experiments to obtain assignments for individual spin systems (i.e. sugars) [51]. A 2D ^{19}F , ^1H HMQC was acquired to identify chemical shifts of pyrimidine sugar protons (H1', H2', and H3') correlated to the 2' fluorine resonance. The experiment was conducted at 25°C on a 500 MHz Varian spectrometer using a $^{19}\text{F}/^1\text{H}$ probe, and ^{19}F decoupling was not used during acquisition (t_2). For this experiment, 2048 complex points were collected in t_2 , and 106 complex points were acquired in t_1 . A sweep width of 5006 Hz was used for both dimensions. The spectrum was processed using NMRPipe, and a cosine bell window function was applied in each dimension. H1', H2', and H3' chemical shifts were identified based on their location in the spectra and their observed couplings. Resonances were given temporary designations until permanently assigned.

2.5 Acquisition of a 2D ^{19}F , ^1H HOESY on the Macugen-HBD complex to identify H1', H2', H3' resonances and obtain sequence-specific information

2D ^{19}F , ^1H heteronuclear Overhauser effect spectroscopy (HOESY) was employed to compliment/confirm the information obtained from the ^{19}F , ^1H HMQC, and it was also used to identify the chemical shift of the H6 or H8 of nucleotides directly following pyrimidines in the 5' to 3' direction in the sequence [52]. A 2D ^{19}F , ^1H HOESY was performed at 25°C on a 500 MHz Varian spectrometer using a $^{19}\text{F}/^1\text{H}$ probe and a mixing time of 300 ms. No ^{19}F decoupling was applied during t_2 (acquisition). The experiment was collected with 352 scans per increment, 160 complex data points in t_1 , and 4096 complex points in t_2 . A sweep width of 7400 Hz was chosen for t_1 , while the sweep width for t_2 was set to 6000 Hz. Using NMRPipe for processing, baseline correction was achieved by polynomial time-domain solvent subtraction. Furthermore, an exponential window function with a line broadening of 5 Hz was applied in the proton dimension while a cosine squared window function was used for the fluorine dimension. The spectrum was compared to the ^{19}F , ^1H HMQC in order to validate the information obtained from that experiment, and it was also used to locate chemical shifts of H6/H8 protons belonging to residues following pyrimidines in the sequence.

2.6 Collection of a 2D ^1H , ^1H NOESY in D_2O on the Macugen-HBD Complex to make sequential assignments

A 2D ^1H , ^1H NOESY was acquired at 25°C in 100% D_2O . The spectrum was acquired on an 800 MHz instrument equipped with a $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ triple resonance cold

probe with z-axis pulsed field gradients [53]. A 200 ms mixing time was utilized, and water suppression was achieved through presaturation. NOESY data was collected with 80 scans per increment, 400 complex points in the t_1 dimension, and 4096 complex points in the t_2 dimension. The spectral width was set to 10000 Hz in the t_1 dimension and 12019 Hz in the t_2 dimension. The data was processed with NMRPipe. Baseline correction was achieved with polynomial time-domain solvent subtraction, and a cosine bell window function was applied in each dimension. Pyrimidine H6-H5 NOEs were identified by their direct overlap with H6-H5 TOCSY cross-peaks. This spectrum was then used in conjunction with the 2D ^1H , ^1H TOCSY, the 2D ^{19}F , ^1H HMQC, and the 2D ^{19}F , ^1H HOESY to examine intra- and inter-residue NOEs and make sequential assignments.

CHAPTER 3

Results and Discussion

3.1 Comparison of traditional assignment strategy for unlabeled nucleic acids with the Macugen assignment process

The basic strategy for assigning unlabeled RNA consists of two phases. First, through-bond experiments can be used to identify resonances belonging to individual spin systems (sugars and bases). Homonuclear correlation experiments such as the ^1H , ^1H COSY and ^1H , ^1H TOCSY can be used to identify nucleobase resonances (i.e. H5/H6) as well as resonances belonging to sugar resonances (H1', H2', H3', etc) [49]. However, through-bond experiments do not provide enough information to confidently assign resonances to specific residues in large, unlabeled RNAs [54]. For example, a resonance may be identified as belonging to an H6 proton, but without the availability of additional methods, this atom cannot be assigned to its individual nucleotide in the sequence. Compounding this problem is the fact that it is difficult to correlate nucleobase protons with their ribose counterparts using scalar techniques (unless the RNA is double-labeled and multi-dimensional NMR techniques are utilized). There has been some success with such experiments as the *J*-scaled COSY for correlating sugar/base protons, but these experiments are restricted to small nucleic acids [55].

To overcome these problems, through-space (dipolar) methods can be employed to examine the specific position of spin systems within the sequence [53]. For example, the ^1H , ^1H NOESY can produce inter-spin system NOEs that can be

used to conduct a base-to-sugar walk. This technique involves following NOEs from nucleobase protons to the intra-residue sugar H1', H2', and H3' atoms. From the sugar atoms, inter-residue NOEs can be examined to continue the 'walk' to the base protons of the following nucleotide in the sequence. As long as there is a known starting point (or if the identity of a residue within the walk can be confirmed), sequence-specific assignments can be made with relative ease assuming the walk is not interrupted. Though NOE-based methods overcome the sequence-specific assignment problems that scalar techniques possess, they lack the confidence and straightforward analysis that are central to through-bond methods. ^1H , ^1H NOESY spectra of large RNAs often exhibit significant overlap, thereby making it difficult or even impossible to perform extensive sugar-to-base walks. Additionally, NOESY spectra are often rather ambiguous; multiple cross-peaks may be seen for the same resonance [49]. As a result, confident assignment of all resonances is often impractical. Homonuclear through-bond techniques are often less ambiguous since they rely on J-couplings between bonded nuclei and because signals do not overlap to the degree observed in NOESY spectra. Assignment of most large, unlabeled RNAs relies on significant contributions from both homonuclear scalar methods and through-space experiments.

Though homonuclear scalar and dipolar methods can be used in combination to compensate for the individual limitations of each other, the information content of these experiments is, nevertheless, often inadequate to achieve near-complete assignment of large, unlabeled molecules with a high degree of certainty. This often results in attempts to isotopically label the RNA of interest or the use of other

techniques to obtain a molecular structure. The situation can become rather complicated if the RNA of interest is limited in quantity or if it cannot be isotopically labeled. One example of such an RNA is Macugen, which can only be obtained in small quantities and in unlabeled form. To assign nucleic acids such as Macugen, strategies need to be developed to overcome the limitations of traditional homonuclear NMR methods. Fortunately, Macugen possesses several chemical modifications which make it more amenable to assignment compared to unmodified counterparts of similar size. Foremost, ^{19}F is a spin-1/2 nucleus and is found at 100% natural abundance, thereby making NMR studies of molecules containing this species possible [53]. ^{19}F also displays excellent chemical shift dispersion and is one of the most NMR-sensitive nuclei, exhibiting a gyromagnetic ratio very close to that of ^1H (the most sensitive) [53]. Because Macugen contains 2'-F pyrimidines, unique NMR experiments can be performed that are not otherwise utilized with assignment of unmodified, unlabeled RNA

While traditional homonuclear scalar correlation experiments (such as the ^1H , ^1H TOCSY) described previously can still be successfully employed to obtain valuable information such as H5/H6 resonances, the fluorine modification of Macugen makes the use of more powerful heteronuclear experiments possible. In these experiments, the fluorine is essentially acting as an additional dimension by which resonances can be separated and identified. For example, a 2D ^{19}F , ^1H heteronuclear multiple quantum coherence (HMQC) spectrum can be used to identify chemical shifts of pyrimidine sugar protons (H1', H2', and H3'). Traditional strategies used to assign unlabeled RNA may take advantage of a homonuclear ^1H , ^1H

TOCSY to help identify sugar protons. While this type of experiment does take advantage of multiple dimensions to “separate” resonances, there are still several limitations to using this type of experiment with large molecules. For instance, all protons experiencing ^1H - ^1H coupling will produce signals, thereby increasing the chances that spectral overlap will be problematic. Through use of the ^{19}F , ^1H HMQC, however, only protons that are coupled to fluorine will appear in the spectrum (rather than all proton-proton coupled resonances). Additionally, the 2'-F modification allows pyrimidine sugar resonances to be easily distinguished from those of purines in scalar experiments. Because Macugen does not contain any purine protons coupled to fluorine, the signals produced in scalar correlation experiments must necessarily come from pyrimidines.

The 2'-F pyrimidine modification carries several other benefits. First, the fluorine imparts a unique germinal coupling to the H2' protons that is absent from unmodified (2'-OH) RNA. H2' resonances in spectra that are not decoupled will exhibit a splitting due to this germinal coupling, and they will be separated by ~50 Hz. Geminal coupling is not observed for the H2' proton in unmodified RNA because the oxygen is not an NMR-active species. Furthermore, 2'-OH proton undergoes rapid exchange and is not bonded to the oxygen long enough to impart a vicinal coupling (via the oxygen) to the H2'. Consequently, the H2' of unmodified RNA experiences only vicinal coupling to the H1' and H3', and these couplings are rather small. For example, the $J_{\text{H1}'\text{-H2}'}$ coupling in unmodified RNA ranges from 1 to 8 Hz depending on the sugar pucker and associated torsion angle [49]. Thus, the rather large and distinctive ^{19}F - ^1H coupling allows for easier assignment of H2' chemical

shifts in Macugen. The 2' fluorine of pyrimidines will also couple to the H1' and H3' protons, inducing a larger splitting in these signals than is normally seen. In Macugen pyrimidines, H3' splitting is observed to be about 27 Hz, whereas the H1' splitting is about 15Hz.

Unfortunately, the enhanced information obtained from heteronuclear scalar experiments utilizing the 2'-F group is still not sufficient to solve the traditional problem of assigning resonances to their sequence-specific nucleotides. For instance, resonances seen in the ^{19}F , ^1H HMQC can be identified as sugar protons, but there is insufficient information at this point to be able to assign these resonances to their respective residues in the sequence. Likewise, there is no way to correlate these sugar resonances with their intra-residue base protons (H5/H6). As with traditional strategies for assigning unlabeled RNA, through-space (dipolar) techniques must still be introduced in order to sequentially assign Macugen. Fortunately, the chemical modifications of Macugen bring unique advantages to the table over traditional unmodified RNA. First, the 2'-F modification can be used to acquire a 2D ^{19}F , ^1H heteronuclear Overhauser effect (HOESY) spectrum. This method identifies resonances that exhibit an NOE to the 2'-F of pyrimidines. In other words, this experiment can provide information about protons that are within ~ 5.0 Å from the fluorine group. It is important to note that NMR of unmodified RNA does not normally make use of the substituent at this position on the ribose; the 2'-OH proton in unmodified RNA undergoes rapid solvent exchange, and it is absent in spectra taken in D_2O . The most significant advantage of performing the ^{19}F , ^1H HOESY is that it can give vital *sequential* information about the position of a pyrimidine within

the sequence. This is because the 2'-F group will exhibit NOEs to the H6/H8 of the following residue in the 5' to 3' direction if the RNA is in A-form conformation (or if the fluorine is positioned to do so in other conformations). Thus, the ^{19}F , ^1H HOESY provides inter-residue information about the H6/H8 resonance belonging to the *next* nucleotide in the sequence. Though similar information can be obtained from the 2D ^1H , ^1H NOESY (via H2'/H3' NOEs to the following residue), the ^{19}F , ^1H HOESY is not subject to the severe overlap that the traditional proton-proton NOESY is. Finally, the ^{19}F , ^1H HOESY can be used to validate the information obtained from the ^{19}F , ^1H HMQC since ribose sugar protons (H1', H2', and H3') are in proximity to the fluorine group.

The 2'-OMe modification on Macugen purines is also of substantial importance, and like the 2'-F, this group has several benefits that make assignment more amenable than unmodified (and unlabeled) RNA. Information derived from this modification is extremely valuable due to the fact that this substituent occupies a position on the ribose that is not normally utilized in NMR experiments (the proton of 2'-OH is exchangeable). Next, the 2'-OMe protons lie between 3 and 4 ppm and will thus show signals in a region of the ^1H , ^1H NOESY where resonances are not normally observed with unmodified RNA. Like the 2'-F, the purine 2'-OMe protons can exhibit NOEs to the H6/H8 of neighboring (3') residues, thus providing information regarding the position in the sequence. The 2'-OMe protons can also exhibit NOE cross-peaks to the H1' and H2' sugar protons, thus providing a means to substantiate these assignments. In addition to looking for NOEs between the sugar protons and the H8 of purines, NOEs can also be found between said protons and the

2'-OMe group. Finally, Macugen also contains a deoxythymine (dT28) which is reverse-linked (3'—3') at the 3' terminus. Unlike traditional RNA that lacks such a residue, distinct signals will be seen which can be attributed to dT28. For instance, the C7 methyl protons of dT28 are found at 1.7 ppm. Because of the unique heteronuclear experiments that can be performed using the 2'-F, the special properties that fluorine imparts (such as the distinct couplings), the advantages of the 2'-OMe groups, and the presence of a terminal deoxy residue, a large deal of Macugen residues were able to be confidently assigned. Importantly, due to these modifications, Macugen is more amenable to the assignment process than unlabeled, unmodified RNA molecules of similar size.

3.2 Identification of pyrimidine H5/H6 resonances from the 2D ^1H , ^1H TOCSY

A 2D ^1H , ^1H TOCSY was first collected to identify pyrimidine H5/H6 resonances. This through-bond experiment correlates multiple proton resonances that are coupled within a spin system. Both C and U exhibit identical spin systems consisting of the coupled H5 and H6 protons. **Figure 3.1** shows the assigned ^1H , ^1H TOCSY spectrum collected on the Macugen-HBD complex, and **Table 3.1** gives the chemical shifts for each assignment. The cross-peaks for these resonances are well-dispersed and occur in a distinct region of the spectrum (~5.0-6.5 ppm for H5 and ~6.5-9.0 ppm for H6), so spectral crowding is not a problem. It should be noted that purine nucleobase resonances cannot be established with this type of experiment since there are no coupled protons in these spin systems (the labile imino/amino protons are not coupled to the carbon-bound protons). Though the pyrimidine H5/H6

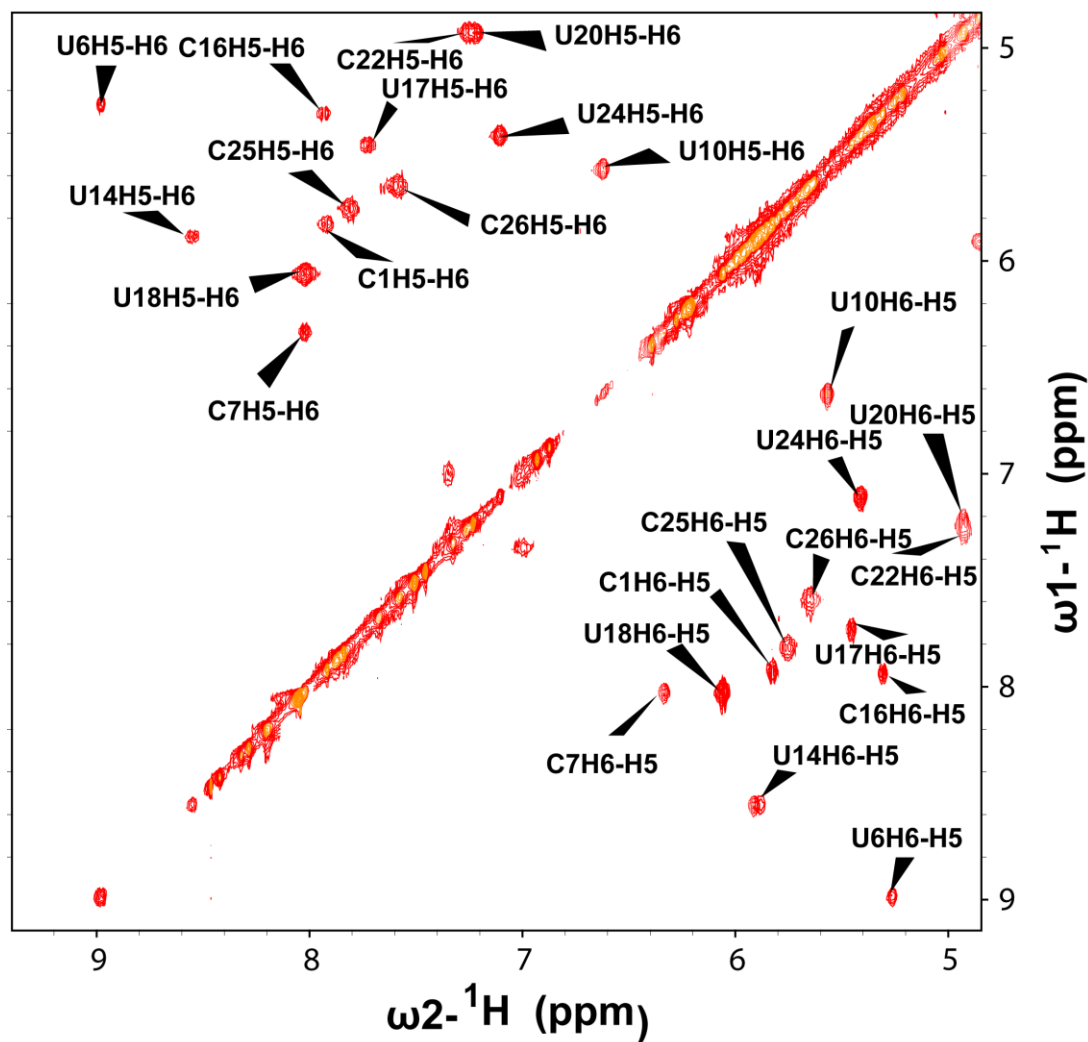


Figure 3.1 2D ^1H , ^1H TOCSY spectrum of the Macugen-HBD complex expanded on pyrimidine H5/H6 resonances. A 2D ^1H , ^1H TOCSY spectrum with a 48 ms mixing time was collected at 500 MHz. All 13 expected H5/H6 peaks are seen on both sides of the diagonal.

Pyrimidine H5/H6 Resonance Assignments from ^1H - ^1H TOCSY		
Assignment	H5 (ppm)	H6 (ppm)
U20	4.911	7.197
U10	5.547	6.598
U24	5.395	7.081
C16	5.287	7.91
U17	5.438	7.705
C26	5.631	7.562
U18	5.809	7.903
C25	5.74	7.791
C7	6.316	8.002
C1	6.039	8.004
U14	5.866	8.537
U6	5.866	8.958
C22	4.90	7.236

Table 3.1 Final pyrimidine H5/H6 assignments in the ^1H - ^1H TOCSY spectrum of the Macugen-HBD complex.

resonances obtained from the ^1H , ^1H TOCSY cannot be assigned to their sequence-specific nucleotide from this spectrum alone, the experiment is necessary for several reasons. First, acquisition of the ^1H , ^1H TOCSY provides resonances to which pyrimidine sugar protons can be correlated to during initial base-to-sugar walks in the ^1H , ^1H NOESY spectrum. Additionally, acquisition of the ^1H , ^1H TOCSY allows the H5/H6 NOESY cross-peaks to be easily located since it can simply be overlaid onto the ^1H , ^1H NOESY spectrum.

3.3 Identification of pyrimidine H1', H2', and H3' resonances from the 2D ^{19}F , ^1H HMQC

Next, a ^{19}F , ^1H HMQC was acquired to obtain resonances for pyrimidine sugar protons, including the H1', H2', and H3'. This experiment correlates protons and fluorine atoms that are coupled within individual spin systems. Because only pyrimidines contain the 2'-F group, no purine sugar resonances will be seen in this experiment. **Figure 3.2** shows the full ^{19}F , ^1H HMQC spectrum with final assignments. There are several notable features of this spectrum as seen in **Figure 3.3**. First, with the exception of U14, H1' protons are located the farthest downfield and the H3' protons are located the farthest upfield (with the H2' protons in between). H2' protons exhibit a splitting of ~50Hz, while H1' and H3' protons are split by 15 Hz and 27 Hz, respectively. Except for U14, all pyrimidine H1' and H2' resonances were located. All 13 fluorine resonances were observed in the spectrum. Additionally, all but five of the 13 pyrimidine H3' resonances were observed. It is

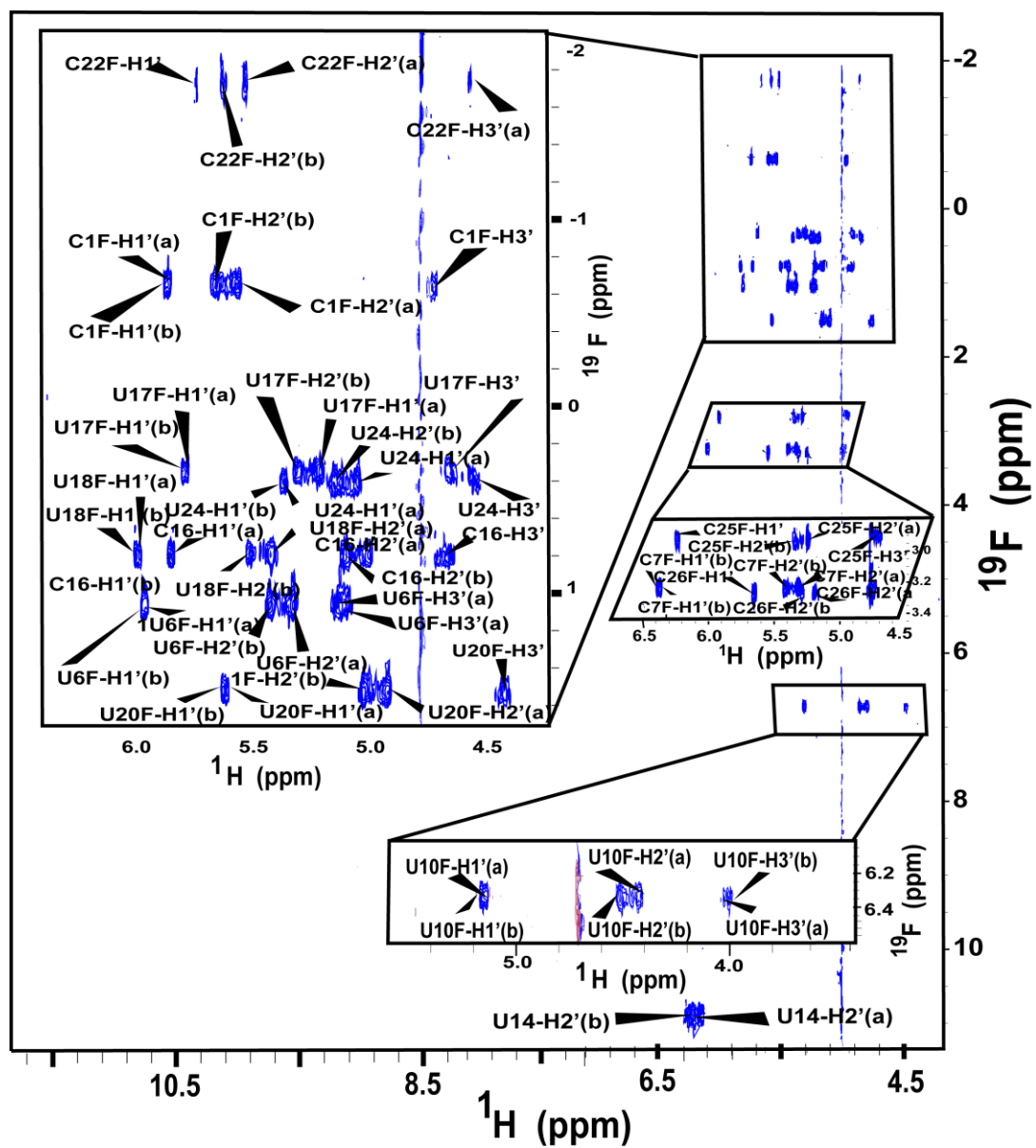


Figure 3.2 ^{19}F - ^1H HMQC collected on the Macugen-HBD complex. The ^{19}F - ^1H HMQC was collected in 100% D_2O on a 500 MHz instrument at 25°C. The spectrum is expanded in three regions and peaks are labeled with final assignments. Additional details are given in **Figure 3.3**.

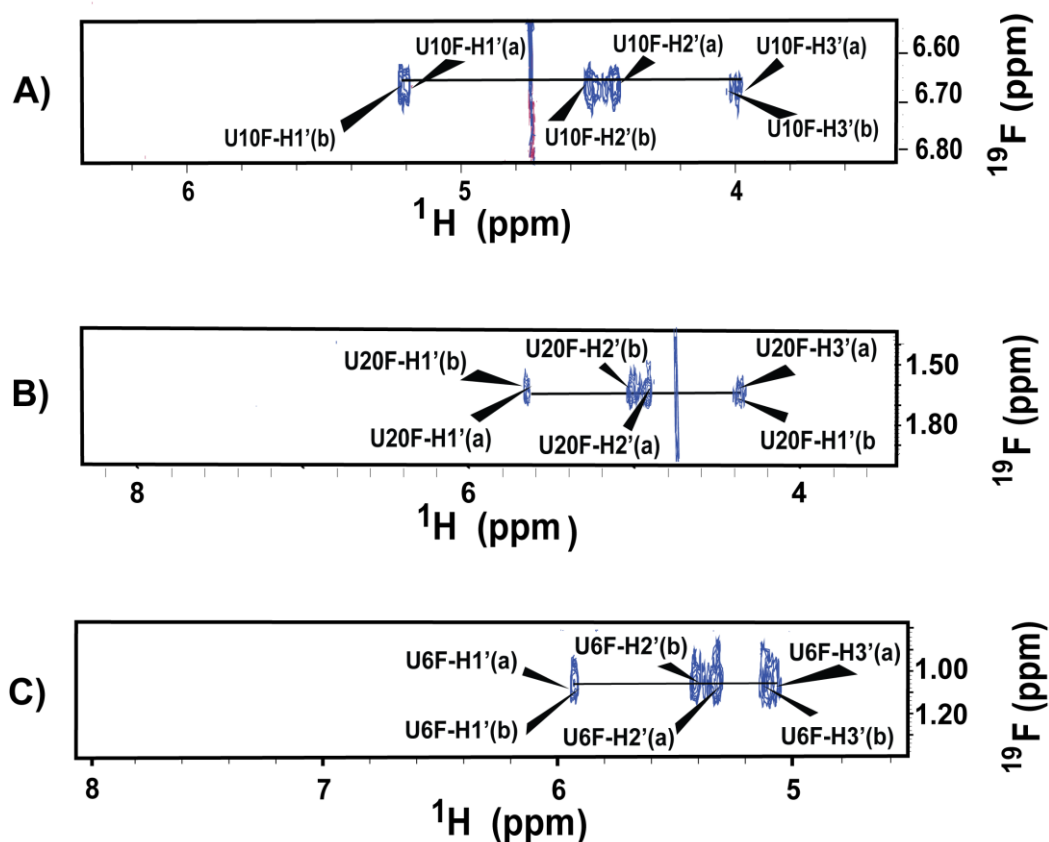


Figure 3.3 Features of the 2D ^{19}F , ^1H HMQC spectra collected on the Macugen-HBD complex. The above figure shows the ^{19}F , ^1H HMQC focused on three individual fluorine resonances. Pyrimidine protons that exhibit scalar coupling to the 2'-F group will appear as cross-peaks correlated to the ^{19}F resonance. Because the experiment was performed with no ^{19}F decoupling during acquisition (t_2), protons will show coupling in this dimension. Coupled peaks are designated 'a' and 'b' to denote the upfield/downfield components. H2' protons will display a coupling to fluorine of ~ 50 Hz to the fluorine. The coupling of fluorine to H2' and H3' protons is smaller; the former displays a coupling of ~ 15 Hz, while the H3' protons exhibit a coupling of ~ 27 Hz. The H1' is usually located farthest upfield, followed by the H2' and H3'.

possible that some H3' resonances were not observed due to overlap with water. Though the sample was in D₂O, the presence of water is unavoidable. Because water has a chemical shift of 4.77 ppm, it is possible its signal overlaps with some H3' resonances (3.9-5.0 ppm). Notably, only one resonance is seen for U14 (assigned through process of elimination), and this was later found to correspond to its H2' proton. The extreme downfield shift of the H2' of U14 is discussed later in the text. The signal-to-noise was not adequate to assign the coupled components of the H3' of C22 in the ¹⁹F, ¹H HMQC, although the coupled pair was clearly defined in the ¹⁹F, ¹H HOESY spectrum. The information obtained from this experiment was extremely valuable. Although resonances were not able to be assigned to their sequence-specific nucleotides from this experiment alone, the H1', H2', and H3' resonances were identified with little ambiguity. Furthermore, identification of pyrimidine sugar proton resonances from the ¹⁹F, ¹H HMQC was critical for correct analysis of subsequent spectra.

3.4 Validation of pyrimidine H1', H2', and H3' resonances and analysis of sequential information from the 2D ¹⁹F, ¹H HOESY

2D ¹⁹F, ¹H heteronuclear Overhauser effect spectroscopy (HOESY) was used to confirm the pyrimidine H1', H2', and H3' sugar resonances obtained from the ¹⁹F, ¹H HMQC, and was also used to identify the chemical shift of the H6 or H8 of residues directly following pyrimidines in the 5' to 3' direction. This experiment identifies protons that are <5Å from the 2'-F group of pyrimidines. **Figure 3.4** shows the 2D ¹⁹F, ¹H HOESY with final assignments. Importantly, ¹⁹F-¹H NOE cross-peaks

can be observed between intra-residue pyrimidine H1', H2', and H3' sugar protons and the 2'-F. Accordingly, this experiment can be used to validate the pyrimidine sugar resonances that were identified through the ^{19}F , ^1H HMQC experiment. All pyrimidine sugar resonances identified in the ^{19}F , ^1H HMQC were also observed in this experiment, making these assignments robust. Additionally, the signal-to-noise of this spectrum is better than that of the ^{19}F , ^1H HMQC, so several additional sugar assignments were made. U14 shows weak peaks for the H1' and H3' protons, and they exhibit the expected splitting (15 Hz and 27 Hz, respectively) for these resonances. Because these peaks lie at distinct chemical shifts, they cannot be attributed to other pyrimidine sugar resonances. The better signal-to-noise of this spectrum compared to the ^{19}F , ^1H HMQC also provided for the assignment of the coupled components of the H3' of C22.

The ^{19}F , ^1H HOESY is a powerful tool because it can provide information about the position of pyrimidines in the sequence. Like unmodified nucleotides, 2'-F pyrimidines exhibit C3'-endo sugar puckers, and RNA containing this group will tend to adopt A-form geometry (especially in helical regions) [56]. In this conformation, the 2'-F group will lie within 5 Å of the H6 or H8 of the following residue in the 5' to 3' direction, and an inter-residue ^{19}F - ^1H NOE should be observed in the ^{19}F , ^1H HOESY. **Figure 3.5** shows the observable intra- and inter-residue NOEs in the ^{19}F , ^1H HOESY. Eleven such NOEs were observed in the spectrum. **Figure 3.6** shows how the ^{19}F , ^1H HOESY can be used to both validate the sugar assignments made from the ^{19}F , ^1H HMQC and to obtain sequential information. In Macugen, it is possible that some pyrimidines in the internal bulge and hairpin do not adopt A-form

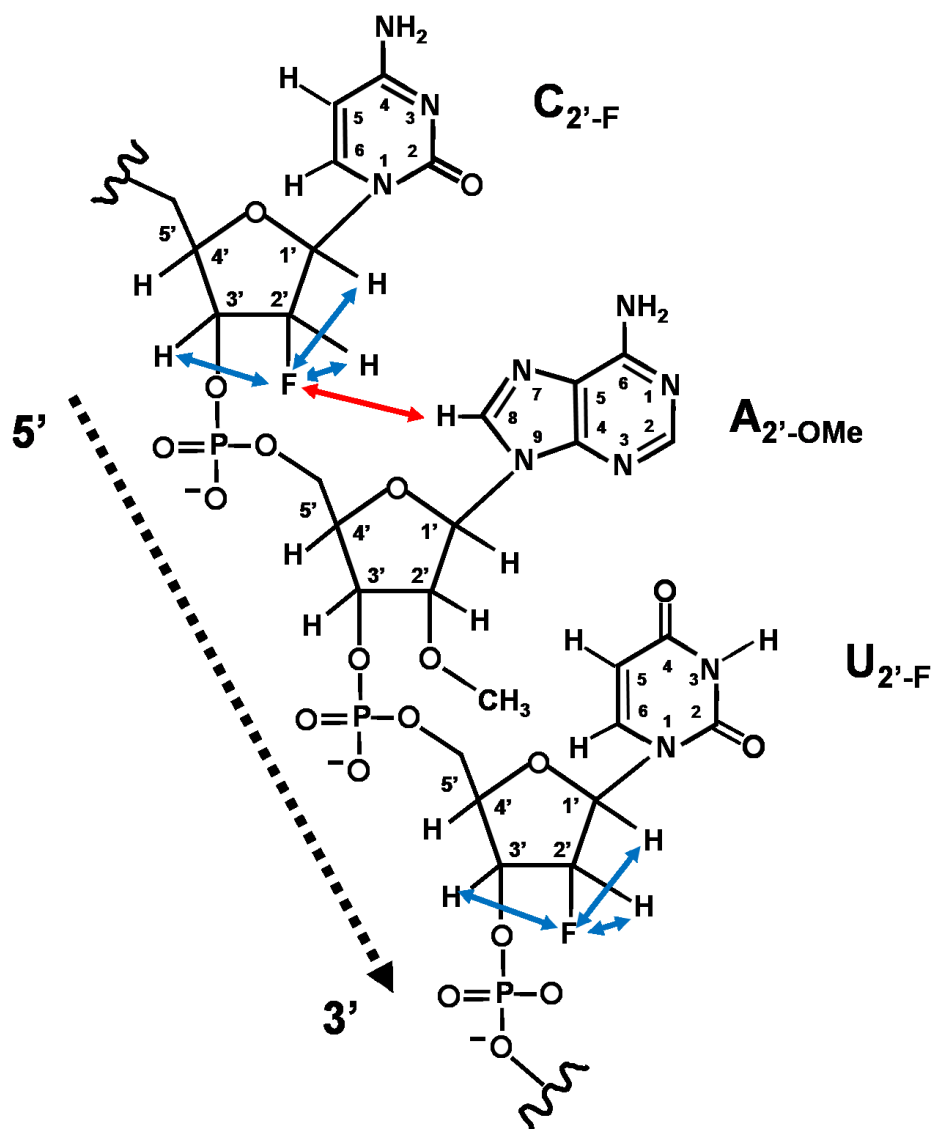


Figure 3.5 Observable Macugen NOEs in the ^{19}F , ^1H HOESY spectrum. Blue lines indicate heteronuclear NOEs between the 2'-F of pyrimidines and intra-residue ribose protons (H1', H2', and H3'). The 2'-F group lies too far away from the intra-residue H6 to exhibit an NOE to this proton, but it can show an NOE to the H6/H8 of the following residue in the 5' to 3' direction (red line). The NOEs were predicted with the assumption that the RNA adopts A-form geometry.

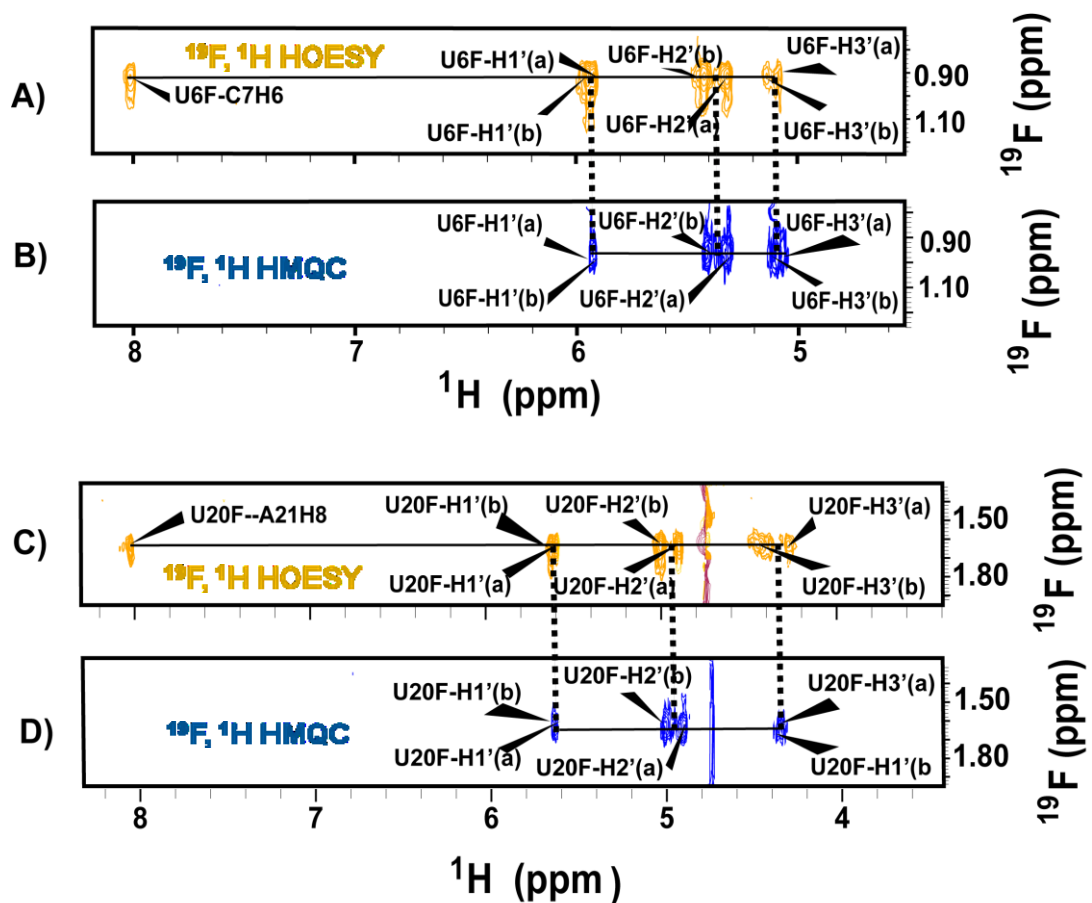


Figure 3.6 Comparison of the 2D ^{19}F , ^1H HMQC (blue) and ^{19}F , ^1H HOESY (yellow) spectra of the Macugen-HBD complex. Panels **A**) and **B**) show the ^{19}F , ^1H HOESY and ^{19}F , ^1H HMQC spectra (respectively) focused on the 2'-F resonance of U6. As with the ^{19}F , ^1H HMQC, intra-residue NOEs between the fluorine and H1'/H2'/H3' nuclei appear in the ^{19}F , ^1H HOESY spectrum due to their proximity to the fluorine. The HOESY experiment also reveals an additional NOE between the fluorine and the H6/H8 of the residue immediately following the pyrimidine in the 5' to 3' direction. In this case, the 2'-F of U6 shows an NOE to the H6 of C7. Panels **C**) and **D**) show an additional example using U20. Because it gives both intra- and inter-residue information, the ^{19}F , ^1H HOESY is valuable during the assignment process.

geometry, and these nucleotides may not show such inter-residue NOEs. Both residues that did not exhibit these NOEs lie in these regions; U14 is located in the hairpin loop while C22 is positioned in the internal bulge. Overall, this information was extremely valuable because it helped to simplify base-to-sugar walks; there was little ambiguity with regards to the base protons of residues following most pyrimidines in the sequence. **Table 3.2** provides the chemical shifts of the resonances assigned in the ^{19}F , ^1H HOESY experiment.

3.5 Analysis of the 2D ^1H , ^1H NOESY in D_2O on the Macugen-HBD complex to make sequential assignments

Though previous experiments were useful for identifying *types* of resonances, until this point in the assignment process, not enough information existed to assign resonances to specific residues of Macugen. A 2D ^1H , ^1H NOESY, in conjunction with the previous experiments, was used to complete the assignment process. A series of sugar-to-base walks were conducted in the ^1H , ^1H NOESY spectrum. Pyrimidine sugar resonances were first correlated to their intra-residue base resonances. Base-to-sugar walks were then used to piece together consecutive pyrimidines and the spectrum was also used to identify H8 resonances of purines following pyrimidines in the sequence. Also, by taking advantage of the unique 2'-OMe purine modifications in Macugen, residues following purines in the sequence were readily identified. Additionally, T28, the sole 2'-deoxy residue (found at the terminus of the nucleic acid), provides additional unique features that were utilized to obtain sequence-specific information from the ^1H , ^1H NOESY.

¹⁹ F- ¹ H HOESY Assignments						
Pyrimidine	F	H1' (a/b) ppm	H2' (a/b) ppm	H3' (a/b) ppm	H6/H8 (n+1) residue (ppm)	
C1	-0.741	5.844/5.860	5.55/5.651		7.653 (G2 H8)	
U6	1.013	5.915/5.942	5.308/5.413	5.07/5.130	8.007 (C7 H6)	
C7	3.157	6.332/6.370	5.297/5.402		8.028 (A8 H8)	
U10	6.663	5.191/5.227	4.439/4.539	3.974/4.026	8.306 (G11 H8)	
U14	10.607	5.403/5.427	6.272/6.384	4.441/4.482		
C16	0.776	5.805/5.828	4.983/5.087	4.569/4.610	7.708 (U17 H6)	
U17	0.368	5.743/5.772	5.193/5.299	4.577/4.636	7.895 (U18 H6)	
U18	0.63	5.946/5.975	5.408/5.506		7.842 (A19 H8)	
U20	1.637	5.605/5.635	4.906/5.009	4.383/4.438	8.03 (A21 H8)	
C22	-1.454	5.709	5.502/5.605	4.521 (not resolved)		
U24	0.423	5.337/5.360	5.069/5.171	4.471/4.541	7.789 (C25 H6)	
C25	3.006	6.191/6.219	5.232/5.339		7.562 (C26 H6)	
C26	3.389	5.616/5.647	5.159/5.261		7.553 (G27 H8)	

Table 3.2 Macugen assignments from the ¹⁹F, ¹H HOESY experiment. a/b refer to upfield/downfield components of doublets (coupled peaks).

Figure 3.7 shows the observable ^1H - ^1H NOEs between atoms involved in sugar-to-base walks in the ^1H , ^1H NOESY spectrum. Protons that lie within 5 Å of each other will show NOEs in this experiment, and the figure assumes that the RNA adopts A-form geometry. As mentioned previously, nucleotides containing 2'-F groups are found in C3'-endo conformations, and will therefore tend to adopt A-form geometry (especially in helical regions) [56]. Likewise, nucleic acids containing 2'-OMe moieties also exhibit this geometry [57]. Therefore, Macugen is similar to unmodified RNA in that it likely adopts this conformation in helical regions. It should be noted that there are a variety of inter-residue ^1H - ^1H NOEs that can be observed when RNA is found in the A-form conformation. The H3', H2', and 2'-OMe protons will show NOEs to the H6 or H8 of the following nucleotide in the sequence in the 5' to 3' direction. This is important because it provides multiple means by which sequential information can be validated. For example, if the H2' resonance of a purine is too overlapped to unambiguously identify, the walk can still be performed as long as either the H3' or 2'-OMe resonances are not obscured. Because there are at least three protons that can exhibit inter-residue NOEs to the next residue in the sequence, the probability of a base-to-sugar walk being interrupted is significantly reduced. Likewise, there are a variety of sugar protons that are within 5 Å of their intra-residue base H6/H8 proton. The H1', H2', and H3' are all close enough to the base proton to exhibit an NOE. Hence, if one of the sugar resonances is overlapped, there are still two more protons that can be used to link sugar resonances to their intra-residue base. Again, this reduces the probability of incomplete base-to-sugar walks.

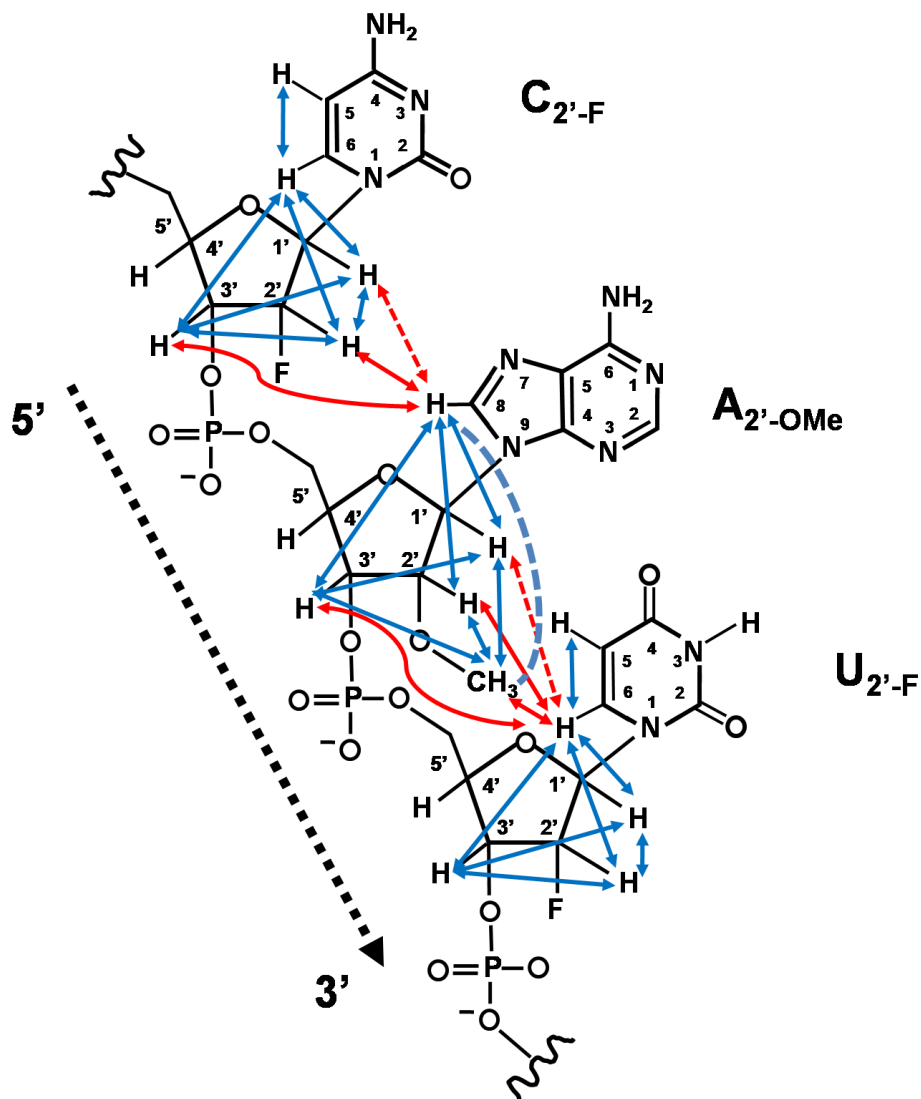


Figure 3.7 Observable ^1H - ^1H NOEs between atoms involved in sugar-to-base walk in the ^1H , ^1H NOESY spectrum (assumes A-form geometry). Intra-residue NOEs are depicted with solid blue arrows connecting the atoms. Solid red arrows depict inter-residue (sequential) NOEs. The dashed blue arrow between the 2'-OMe of the adenosine and the intra-residue H8 indicates an NOE that likely results from spin diffusion (due to the nuclei being separated by more than 5 Å). Similarly, inter-residue NOEs between the H1' and the H6/H8 of the following (5' to 3') residue are also likely due to spin diffusion, and these NOEs are indicated with dashed red lines.

Figure 3.8 shows the distances between intra- and inter-residue base protons (H6 or H8) and H1'/H2' sugar protons, while **Figure 3.9** shows the distances between intra- and inter-residue base protons (H6 or H8) and H3'/2'-OMe sugar atoms.

NOEs can also be observed between protons if spin diffusion is occurring. Spin diffusion is a process that results in an apparent NOE between atoms that may be farther from each other than the 5 Å limit for NOE formation [58]. For example, even though the 2'-OMe is about 5.2 Å from its intra-residue H6 base proton, an NOE was observed between these nuclei in the spectra (**Figure 3.9**). Additionally, spin diffusion NOEs may be seen between H1' protons and the H6/H8 of following residues. Measurements indicate that this distance is ~5 Å, and it is possible that some NOEs observed between these nuclei may be the result of spin diffusion if the distance is >5 Å (**Figure 3.8**). Spin diffusion can occur in NOESY experiments with long mixing times, and the 200 ms mixing time for the ¹H, ¹H NOESY experiment provided ample time for spin diffusion to occur. Spin diffusion NOEs between the 2'-OMe and its intra-residue H8 were observed in the ¹H, ¹H NOESY spectrum. Though spin diffusion is sometimes unwanted, it is useful in the assignment of Macugen because it provides another means by which intra and inter-residue NOEs can be validated. For example, if the H3' of a purine is severely overlapped, the 2'-OMe or H1' can still be used to link the sugar with the intra-residue base. Likewise, if pyrimidine H2' and H3' resonances are severely overlapped, a spin diffusion NOE between its H1' and the H6/H8 of the following residue can avoid an interruption in the sugar-to-base walk.

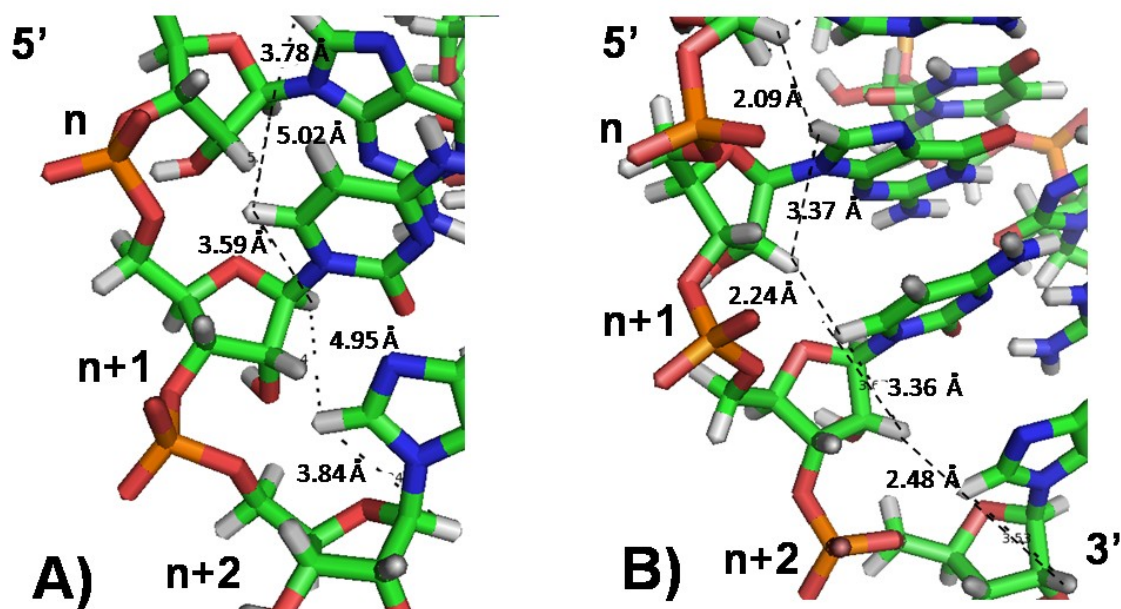


Figure 3.8 Intra-/inter-residue distances between base protons (H6 or H8) and H1'/H2' atoms in A-form RNA. Panel **A**) shows the distance (in Å) between several H1' atoms in an A-form RNA helix and their intra-residue H6/H8 [57]. Also shown are the distances between H1' atoms and the H6/H8 of adjacent residues in the 3' direction. Intra-residue distances range from ~3.5-4.0 Å, whereas inter-residue measurements average ~5.0 Å. In the Macugen-HBD complex, observed inter-residue proton-proton NOEs between H1' atoms and the H6/H8 of following residues may arise from spin diffusion. Panel **B**) shows distances between H2' atoms and their own intra-residue H6/H8, as well as distances between H2' groups and the H6/H8 of adjacent residues in the 3' direction. The A-form RNA model used for these measurements is the same as that used for H1' distances. These intra-residue distances average ~2.1-2.5 Å, and inter-residue distances range from 3.5 to 3.8 Å.

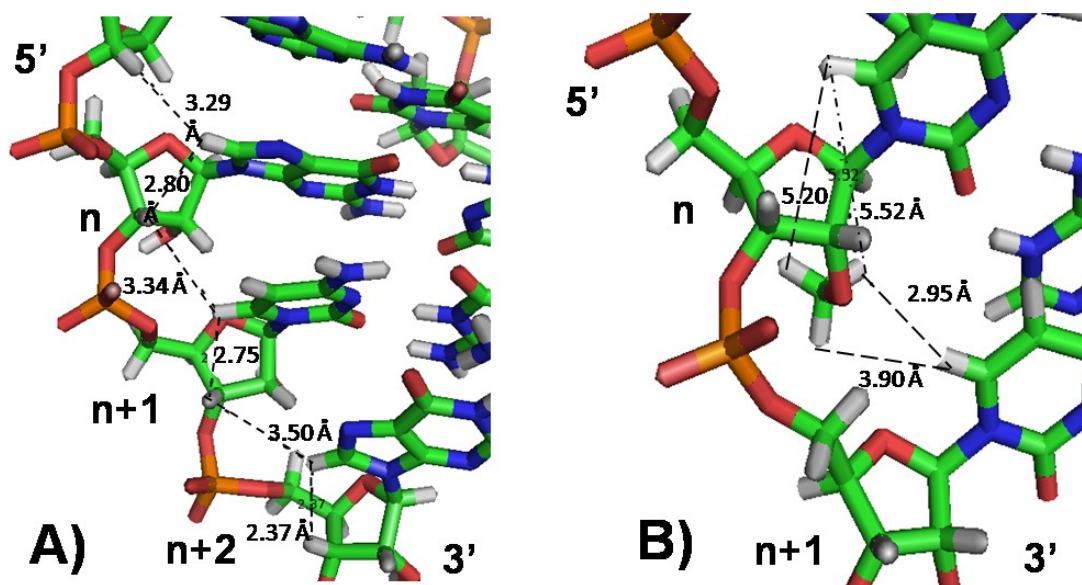


Figure 3.9 Intra-/inter-residue distances between base protons (H6 or H8) and H3'/2'-OMe groups in A-form RNA. Panel **A**) shows the distances (in Å) between several H3' atoms in an A-form helix and their intra-residue H6/H8. The RNA used for these measurements is the same one used in **Figure 3.8** [59]. Also shown are the distances between H3' atoms and the H6/H8 of adjacent residues in the 3' direction. Intra-residue distances range from ~2.3-2.8 Å, whereas inter-residue measurements range from ~3.3-3.5 Å. Macugen residues adopting an A-form (C3' endo) geometry should produce observable intra- and inter-residue NOEs between H3' atoms and base protons. Panel **B**) shows distances between 2-OMe protons in A-form RNA and their own intra-residue H6, as well as distances between the 2'-OMe atoms and the H6 of the following residue in the 3' direction. These intra-residue distances average ~5.35 Å, and inter-residue measurements average about 3.4 Å. Observed intra-residue proton-proton NOEs between the H8 and 2'-OMe protons result from spin diffusion.

Pyrimidine intra-residue H5/H6 NOE cross-peaks were first located in the spectrum by overlaying the 2D ^1H , ^1H TOCSY onto the 2D ^1H , ^1H NOESY. H5/H6 NOE cross-peaks will directly overlap with the H5/H6 TOCSY peaks. **Figure 3.10** shows the overlaid spectra. This established pyrimidine H6 chemical shifts, which are used extensively during the sugar-to-base walk. The next step was to correlate pyrimidine sugar H1', H2', and H3' resonances with their intra-residue base protons to form complete sets of pyrimidine resonances. This was done by searching for H1', H2', and H3' resonances (identified from the HMQC and HOESY) along each pyrimidine H6 chemical shift since all three sugar resonances can exhibit NOEs to the base H6 proton. H1', H2', and H3' protons may also have an NOE cross-peak with the H6 proton of the *following* residue if it happens to be a pyrimidine, so caution must be used to ensure that sugar resonances are not correlated to the wrong H6. Use of the ^{19}F , ^1H HOESY is invaluable for this procedure since it gives information about the chemical shift of the H6/H8 of the following residue. The ribose protons of U14 and C22 did not show NOEs to a following residue, so correlation of their sugar resonances with the intra-residue base H6 was simplified. All sugar resonances were able to be correlated to their intra-residue base protons by this method. **Figure 3.11** and **Figure 3.12** provide two examples of how combined use of the ^1H , ^1H TOCSY, ^{19}F , ^1H HMQC, ^{19}F , ^1H HOESY, and ^1H , ^1H NOESY allowed for intra-residue correlation of pyrimidine sugar resonances with base H6/H5 protons.

To create robust footholds for extensive sugar-to-base walks, inter-residue NOEs were established for consecutive pyrimidines. Additionally, a valuable

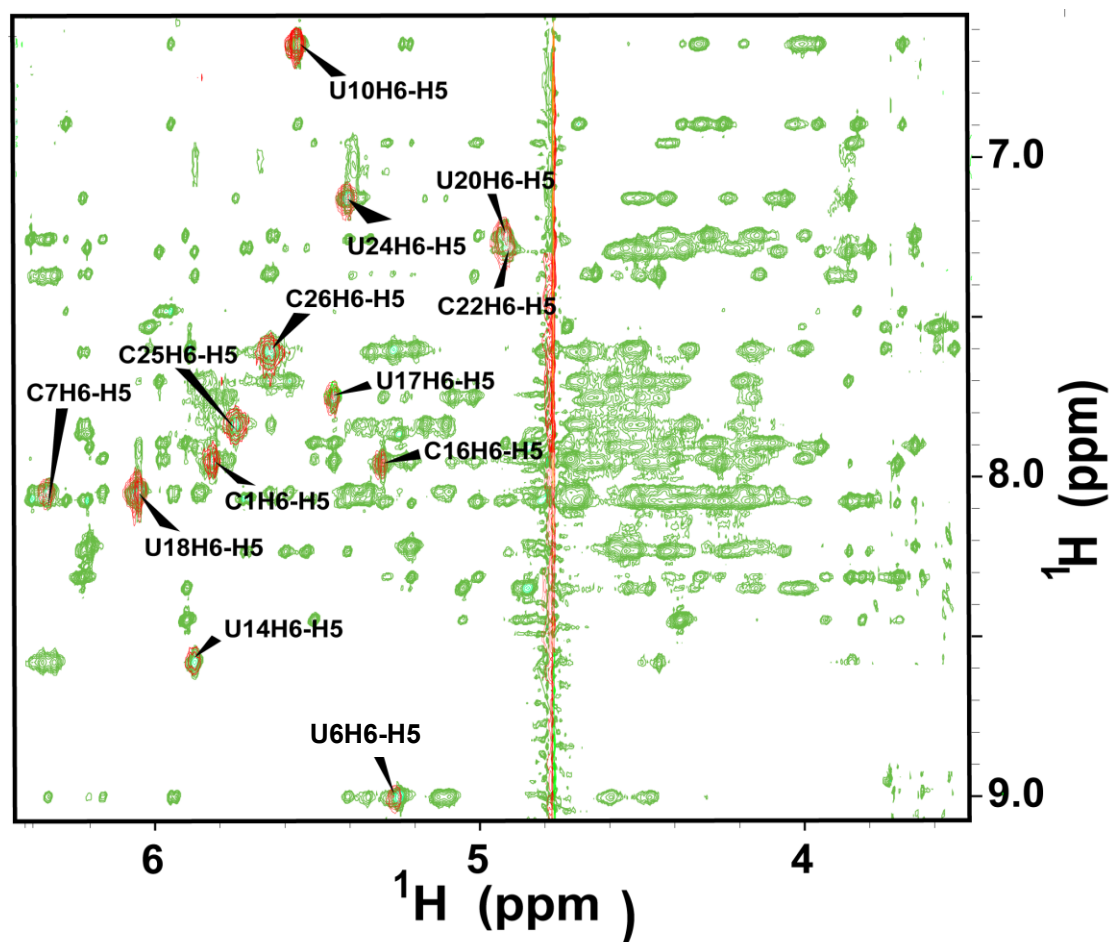


Figure 3.10 Spectral overlay of the 2D ^1H , ^1H TOCSY (red) onto ^1H , ^1H NOESY (green) in the sugar-to-base region of the Macugen-HBD complex. Both spectra of the Macugen-HBD complex were collected at 25°C in 100% D_2O . The NOESY was acquired on an 800 MHz instrument using a 200 ms mixing time and presaturation water suppression. Pyrimidine H6-H5 NOEs are identified by their direct overlap with H6-H5 TOCSY cross-peaks.

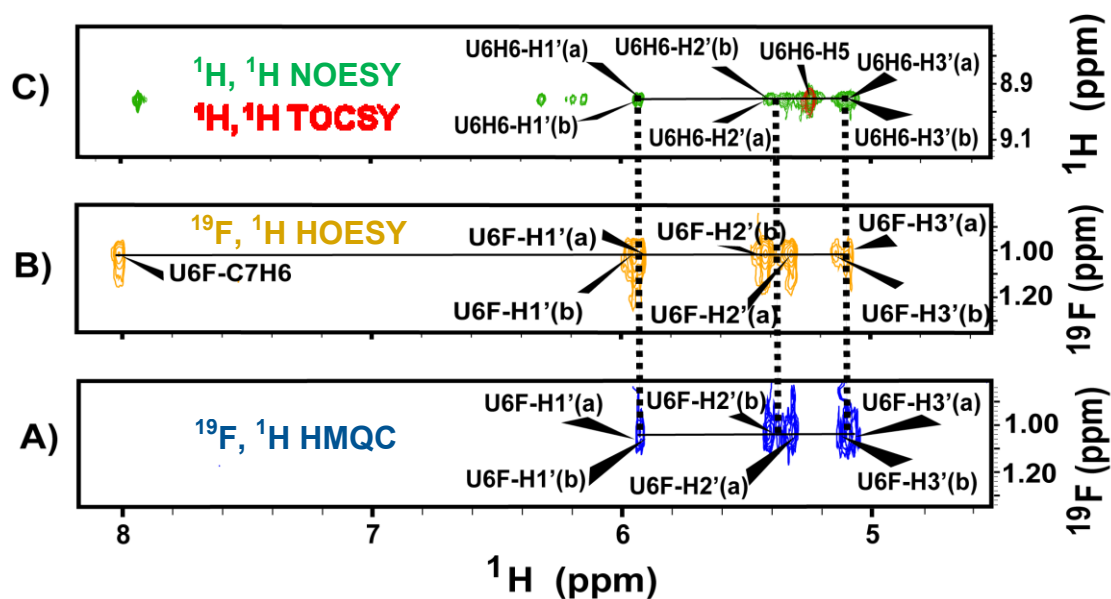


Figure 3.11 Combined use of 2D ^{19}F , ^1H HMQC, ^{19}F , ^1H , ^1H HOESY, ^1H , ^1H NOESY, and ^1H , ^1H TOCSY collected on the Macugen-HBD complex to correlate pyrimidine sugar resonances with intra-residue base protons **A)** The region of the ^{19}F , ^1H HMQC spectrum of the Macugen-HBD complex displaying H1', H2', and H3' resonances of U6 correlated to the 2'-F **B)** Region of the ^{19}F , ^1H HOESY spectrum showing intra-residue NOEs between the 2'-F and the H1', H2', and H3' of the pyrimidine. The H6 chemical shift of the residue following U6 in the 5' to 3' direction (C7) is also observed in this spectrum. **C)** The H6-H5 cross-peaks in the NOESY spectrum (green) were identified by overlaying the TOCSY (red). The NOESY spectrum was then examined for NOEs between the H6 protons of U6 and its intra-residue H1', H2' and H3' sugar protons.

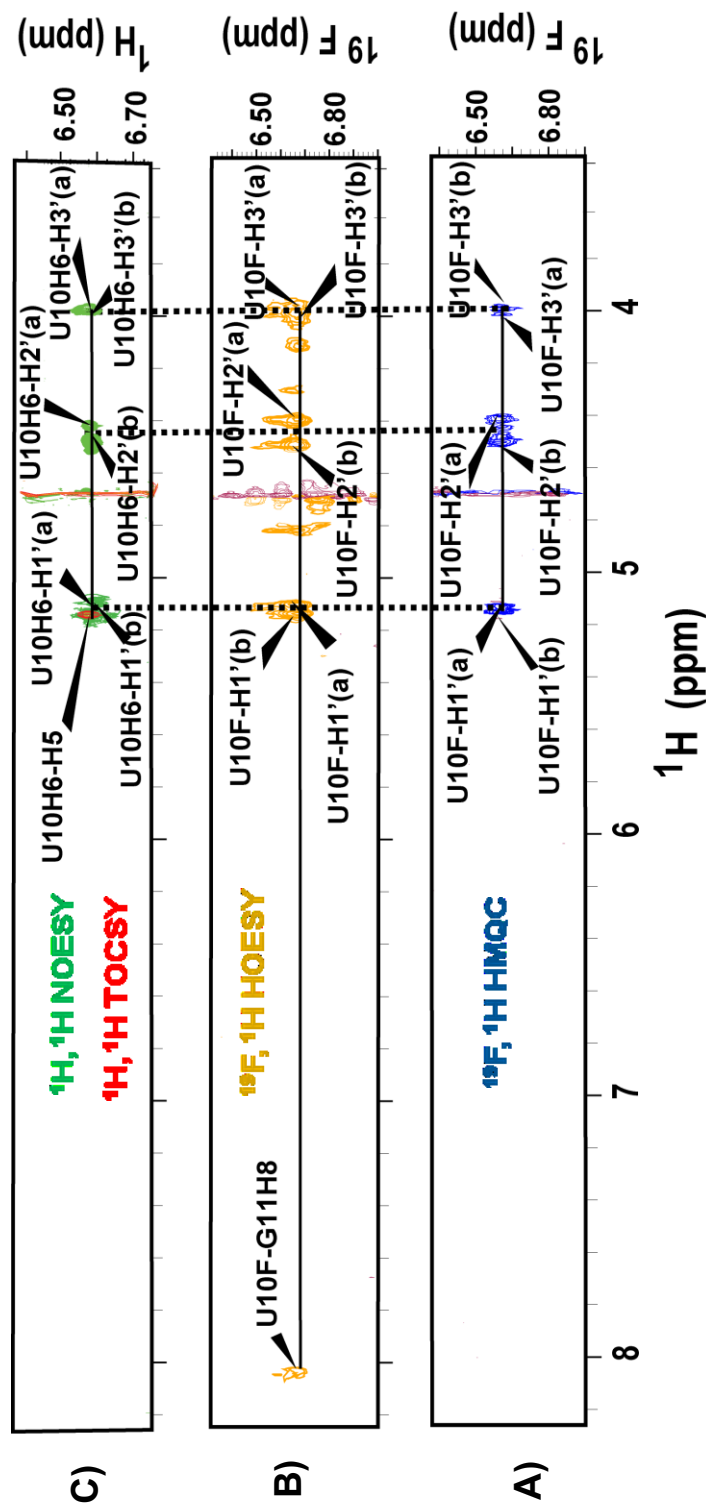


Figure 3.12 Another example of combined use of 2D ^{19}F , ^1H HMQC, ^{19}F , ^1H , ^1H HOESY, ^1H , ^1H NOESY, and ^1H , ^1H TOCSY collected on the Macugen-HBD complex to correlate sugar resonances with intra-residue base protons **A)** A region of the ^{19}F , ^1H HMQC spectrum of the Macugen-HBD complex displaying H1', H2', and H3' resonances of U10 correlated to the 2'-F. **B)** ^{19}F , ^1H HOESY spectrum showing intra-residue NOEs between the 2'-F and the H1', H2', and H3' of U10. (CONTINUED)

(Figure 3.12 continued) The H8 chemical shift of the residue following this pyrimidine in the 5' to 3' direction (G11) is also observed. **C)** The ^1H - ^1H NOESY spectrum (green) was examined for intra-residue NOEs between the H6 of U10 and its H1', H2', and H3' protons.

foothold was established by assigning the resonances of G27 and dT28. Because dT28 contains a deoxy residue, it has unique methyl resonances from the H7, and its H2'/H2'' resonances can easily be found in the TOCSY and NOESY. **Figure 3.13** shows the assignment of G27 and dT28. Piecing together all consecutive pyrimidines was done with relative ease due to the fact that all sugar resonances had been defined through the HOESY/HMQC and because the HOESY reveals the chemical shift of the H6/H8 of the following residue. **Figure 3.14** provides an example of how pyrimidine-pyrimidine connectivities were established using the ^1H , ^1H NOESY. Finally, pyrimidines followed by purines were linked together using identified pyrimidine sugar resonances and information from the HOESY. At this point, the 2'-OMe group was used to establish purine-pyrimidine links as well as purine-purine links. **Figure 3.15** shows how the 2'-OMe was used for sugar-to-base walks.

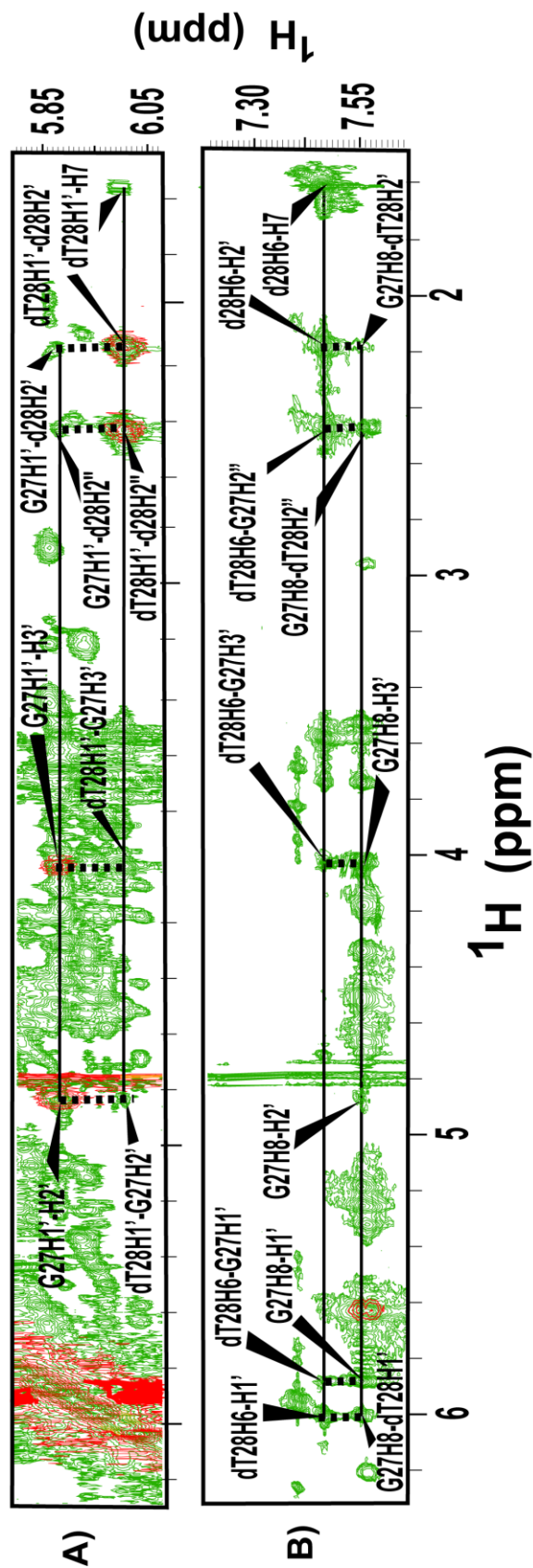


Figure 3.13 Inter-residue NOEs between G27 and dT28. Panel A) shows part of the ^1H , ^1H TOCSY spectrum (red) overlaid onto the ^1H , ^1H NOESY spectrum (green). Being the only deoxy residue in Macugen, dT28 shows distinctive NOEs between the $\text{H}2' - \text{H}1'$ and between $\text{H}2'' - \text{H}1'$. These NOEs are confirmed by the presence of directly-overlapping TOCSY peaks. Because both the $\text{H}2'$ and $\text{H}2''$ are coupled to the $\text{H}1'$, they show correlations to the $\text{H}1'$ in the TOCSY spectrum. Additionally, the $\text{H}1'$ of dT28 exhibits an NOE its methyl proton. Panel A) also shows inter-residue NOEs between the $\text{H}1'$ of G27 and the $\text{H}2'/\text{H}2''$ of dT28. This is expected due to the reverse linkage ($3' \rightarrow 3'$) (CONTINUED)

(Figure 3.13 continued) between these residues. Finally, this panel also shows intra-residue NOEs (as well as TOCSY peaks) between the H1' and H2'/H3' of G27.

Panel **B)** shows intra-residue NOEs between the H6 of G27 and its own H1' and H2' protons. Additionally, the H6 of dT28 exhibits intra-residue NOEs to its H1', H2', and H2'' protons. Inter-residue NOEs are seen between the H8 of G27 and the H1' /H2'/H2'' of dT28. Additional inter-residue NOEs are observed between the H6 of dT28 and the H1'/H2'/H3' of G27.

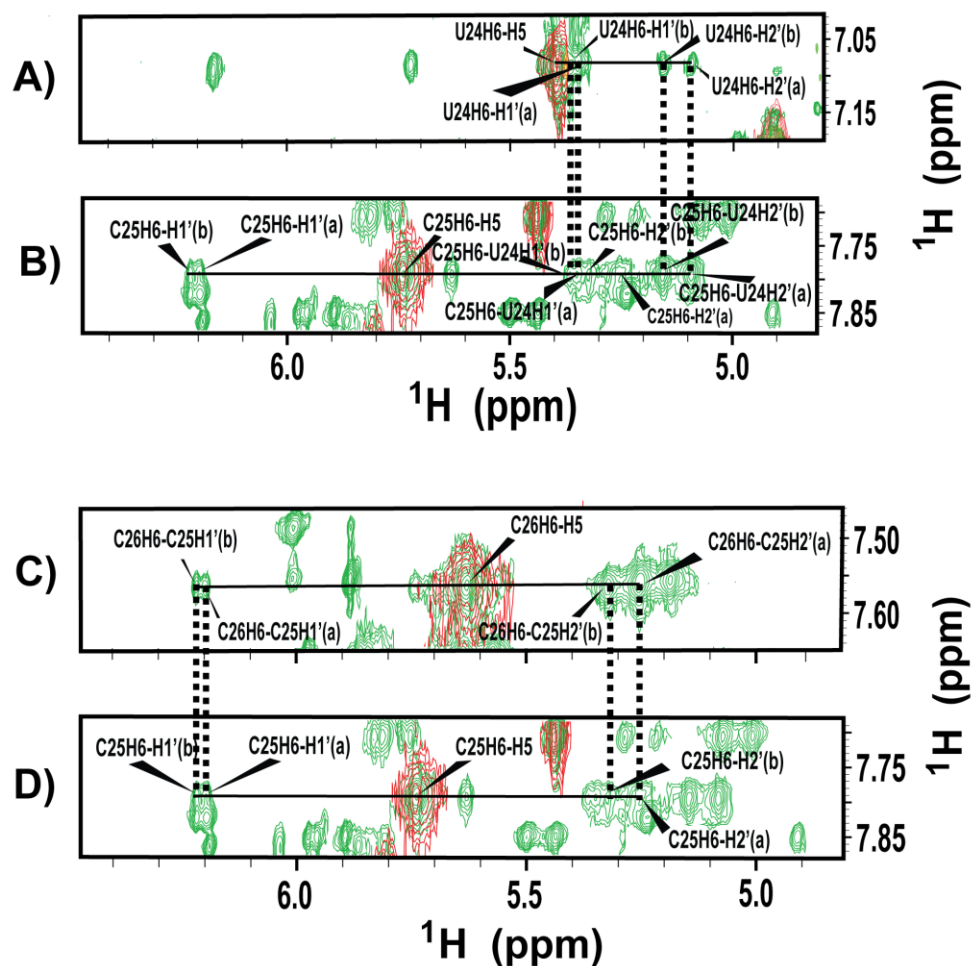


Figure 3.14 Use of inter-nucleotide ^1H - ^1H NOEs to establish pyrimidine-pyrimidine connectivities. Panel **A**) shows the ^1H , ^1H TOCSY (red) overlaid onto the ^1H , ^1H NOESY (green) expanded on a single H6/H5 resonance belonging to U24. H1'/H2' protons show intra-residue NOEs to the H6 (solid line). **B**) The chemical shift of the H6 was shown from the ^{19}H , ^1H HOESY to be 7.79 ppm, corresponding to the H6 of C25. As expected, NOEs are seen between the H6 of C25 and the H1'/H2' of U24 (dotted lines). Intra-residue NOEs are seen between the H6 of C25 and its H1'/H2' protons (solid line) **C**) The H6/H8 chemical shift of the residue following C25 in the sequence is t 7.55 ppm (from the HOESY), corresponding to the (CONTINUED)

(Figure 3.14 continued) H6 of C26. **D** The H1'/H2' of C25 show inter-residue NOEs to the H6 of C26 (dotted lines). Completing the walk, the H6 of C26 has NOE cross-peaks to its intra-residue H1'/H2' atoms.

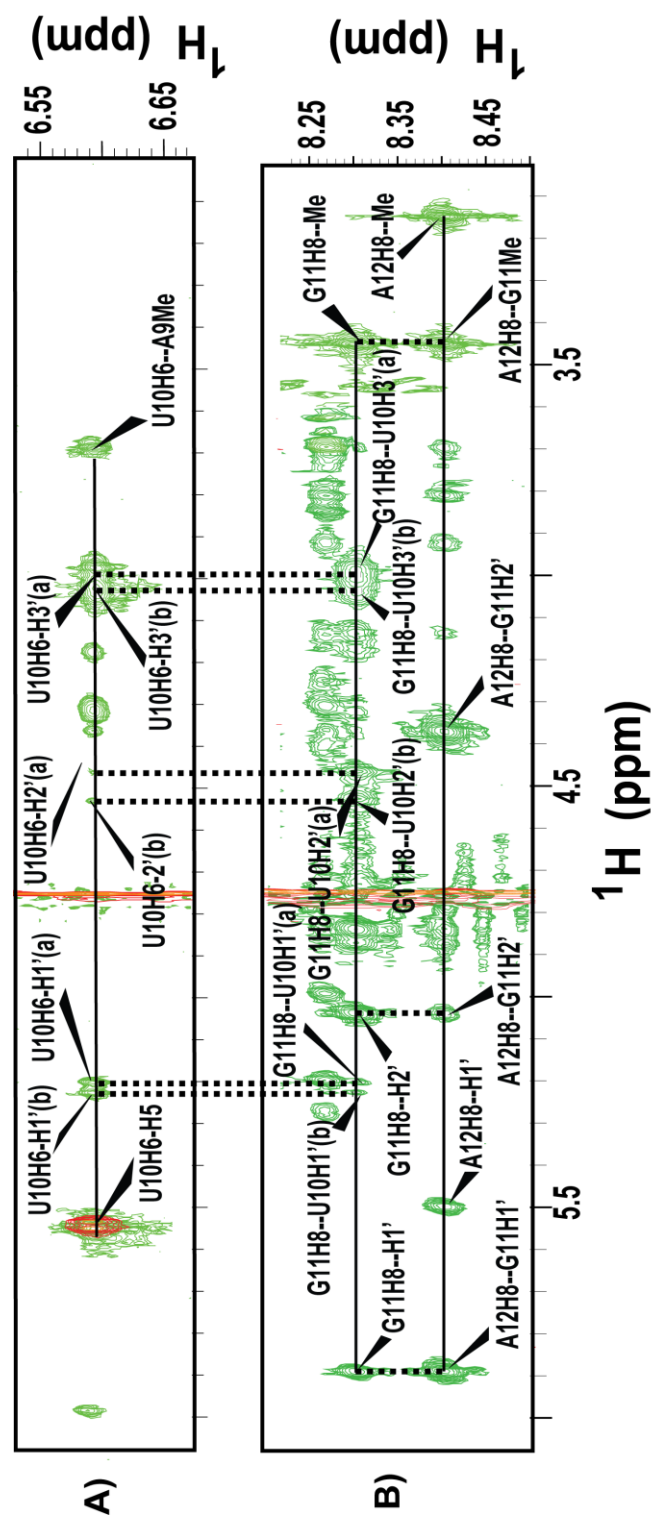


Figure 3.15 Establishing pyrimidine-purine and purine-purine connectivities through sequential NOEs. Panel **A**) shows the ${}^1\text{H}$, ${}^1\text{H}$ TOCSY (red) overlaid onto the ${}^1\text{H}$, ${}^1\text{H}$ NOESY (green). The spectra are expanded on a single pyrimidine H6 resonance (U10). Examination of the ${}^{19}\text{F}$ - ${}^1\text{H}$ HOESY revealed the H6/H8 chemical shift of the residue immediately following pyrimidine ii in the 5'-3' direction to be at 8.3 ppm. There is no H6 located at that shift, so it must belong to a purine H8. In Panel **B**), the H1'/H2'/H3' of U10 show NOEs to this H8 (dotted lines between panels), and it was assigned (CONTINUED)

(Figure 3.15 continued) to G11. Continuing the walk, the H8 of G11 shows NOEs to its intra-residue H1', H2', and 2'-OMe atoms, and the 2'-OMe of G11 was located via its intra-residue NOEs to the H1' and H2' (not shown) as well as its spin diffusion NOE to the H8 (shown). The spectrum was examined for inter-residue NOEs between the 2'-OMe protons of G11 and the H6/H8 of the following (5' to 3') residue, and they were located at 8.4 ppm (shown with dotted lines within panel **B**). This corresponds to the H8 chemical shift of A12. Completing this stretch of the base-to-sugar walk, the H8 of A12 exhibits intra-residue NOEs to its own H1' and H2' protons. Thus, combined use of the intra- and inter-residue NOEs allowed for the establishment of pyrimidine-purine and purine-purine connectivities.

3.6 Conclusions

This work led to the assignment of all but four residues of Macugen. These nucleotides lie in the internal bulge and the hairpin loop, and are probably in regions that do not exhibit A-form geometry. Additionally, an interesting feature is seen in U14. Its H2' resonances lie the farthest downfield—contrary to the usual pattern where H1' protons are found shifted the furthest downfield. This could be due to a unique conformation of this residue since the H2' protons are likely experiencing the most electronegative environment. **Figure 3.16** shows final NOE connectivities from sugar-to-base walks, and **Table 3.3** gives the final assigned chemical shifts for all assigned residues.

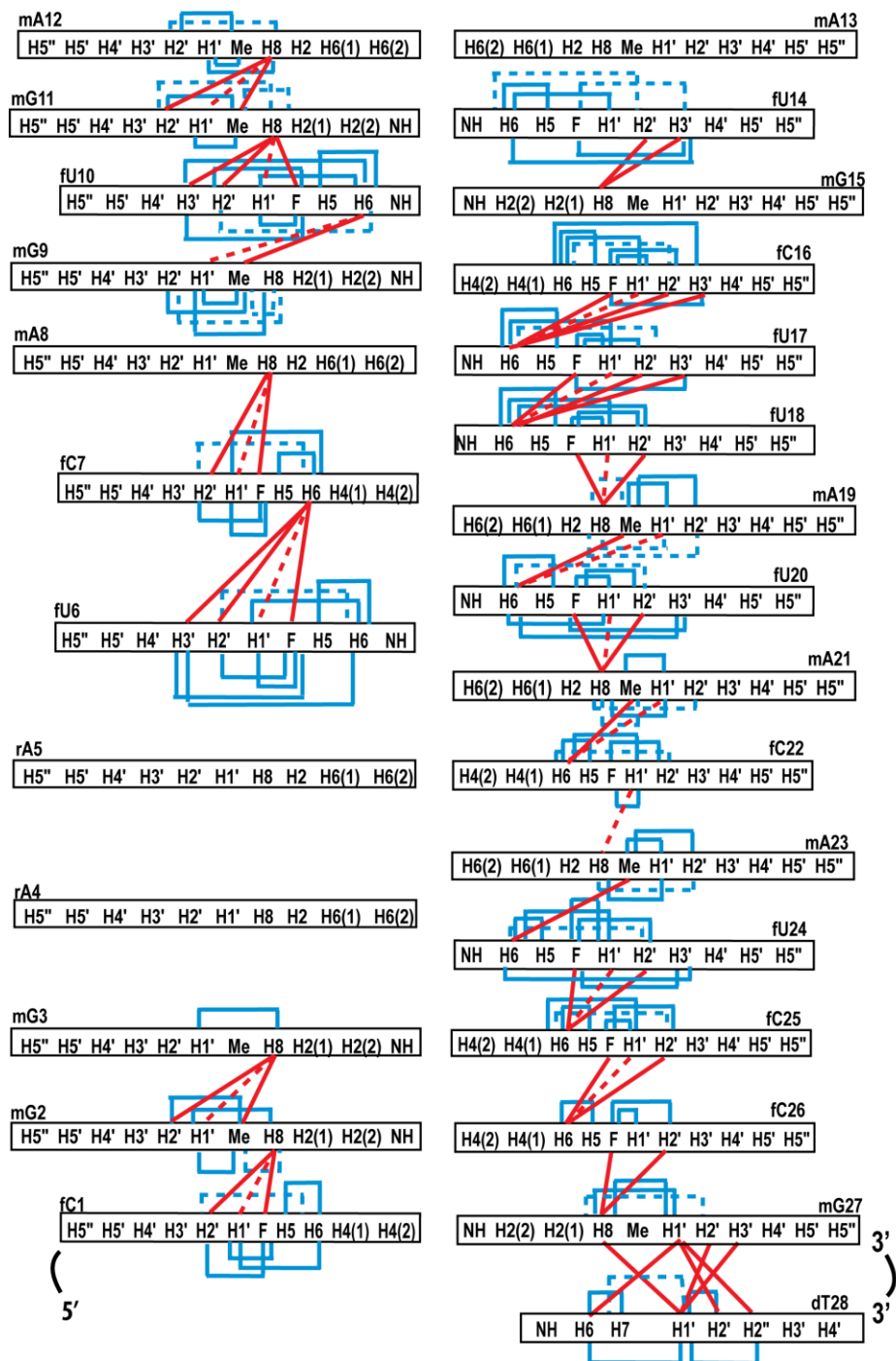


Figure 3.16 Diagram of observed intra-molecular Macugen ^1H - ^1H and ^1H - ^{19}F NOEs in the Macugen-HBD complex. Blue lines indicate intra-residue NOEs and red lines indicate inter-residue NOEs. Dashed lines indicate NOEs likely from spin diffusion.

Base	Sugar Assignments				Base Assignments	
	H1' (a/b) ppm	H2' (a/b) ppm	H3' (a/b) ppm	2'-OMe ppm	H5 ppm	H6/H8 ppm
fc1	5.84/5.866	5.573/5.638			6.039	8.004
mG2	5.974	4.577		3.853		7.655
mG3	5.867					7.251
rA4						
rA5						
fU6	5.922/5.939	5.330/5.393	5.081/5.111		5.866	8.958
fc7	6.34/6.366	5.329/5.396			6.316	8.002
mA8						8.025
mG9	5.939	5.549		3.687		6.852
fU10	5.204/5.227	4.465/4.529	3.952/3.997		5.547	6.598
mG11	5.891	5.039		3.452		8.303
mA12	5.498	4.37		3.148		8.404
mA13						
fU14	5.39/5.414	6.298/6.369	4.44/4.49		5.866	8.537
mG15						7.322
fc16	5.813/5.831	5.006/5.072	4.562/4.616		5.287	7.91
fU17	5.753/5.772	5.216/5.285	4.582/4.643		5.438	7.705
fU18	5.957/5.975	5.437/5.500			5.809	7.903
mA19	5.895	4.91		3.648		7.849
fU20	5.615/5.634	4.928/4.996	4.388/4.444		4.911	7.197
mA21	6.198			3.791		8.031
fc22	5.697/5.715	5.522/5.588	4.497/4.535		4.90	7.236
mA23	6.205	5.395				8.188
fU24	5.348/5.365	5.096/5.156	4.4804.529		5.395	7.081
fc25	6.197/6.216	5.254/5.323			5.74	7.791
fc26	5.624/5.644	5.186/5.253			5.631	7.562
mG27	5.882	3.992	4.03			7.555
dT28	6.006	2.134 (H2')/2.430 (H2'')		1.68 (H7)		7.486

Table 3.3 Final Macugen assignments in the Macugen-HBD complex. Assignments are grouped by sugar/base. Blacked-out cells indicate that the residue does not contain that particular group. Empty cells indicate that no assignment was able to be made for that particular atom. a/b refer to upfield/downfield components of doublets.

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APPENDIX

List of Directories and Filenames

A.1 Original 2D NMR data collected on the Macugen-HBD complex is in directory:

/libby/spectra/hbd-mac/092008/

hbdmac_tocsy_48ms_100408_ael

hbdmac_19fhmqc_stoppedat214_72hrs_092208_ael

hbdmac_hoesy_300ms_101008_ael

hbdmac_noesy200ms_d2o_800_cold_100608v2_ael

A.2 Original Sparky project used to assign Macugen in the complex is in directory:

/libby/sparky/projects/hbdmac/thesis/

libby_thesis_macugen_assignment_final_080210_ael