Effects of Metal Ions and Loop Stability on the Structure and Function of the T Box
Antiterminator RNA and its complex with Model tRNA

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This dissertation titled
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Antiterminator RNA and its complex with Model tRNA

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ABSTRACT

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Effects of Metal Ions and Loop Stability on the Structure and Function of the T Box

Antiterminator RNA and its complex with Model tRNA (210 pp.)

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The T box antitermination mechanism is a novel transcription regulatory system found in many Gram-positive bacteria. The mechanism serves to regulate aminoacyl tRNA synthetases, amino acid transport genes and amino acid biosynthesis genes. The genes regulated by this system are characterized by highly conserved primary and secondary structural elements in the 5’ untranslated region. Two mutually exclusive secondary structures can form in the 5’ untranslated region; the antiterminator, stabilized by uncharged tRNA binding, leads to transcription read through and full synthesis of the gene. The formation of the terminator element, in the absence of uncharged tRNA leads to transcription termination. The antiterminator consists of two helices, A1 and A2 separated by a seven-nucleotide bulge and a closing loop in the A2 helix.

This study investigated the effects of the loop region and Mg$^{2+}$ on the structure and functions of the antiterminator and microhelix tRNA model. The use of computational, spectroscopic and molecular biology techniques demonstrated that the loop region had an effect on the structure and stability of the antiterminator and microhelix RNA but did not change the overall functions of the antiterminator model.

A series of antiterminator models and microhelix RNA models were constructed with a substitution of well-characterized stable GNRA and UNCG tetraloops. A part from these mutations, the wild type antiterminators AM2 with a loop sequence AAUCA and the GlyQS (Glycine synthetase) with a loop sequence (GAAC) that is not a classified stable loop were also investigated. Using Mfold thermodynamic stability and UV
monitored thermal denaturation, it was demonstrated that the loop region contributes significantly to the stability of the antiterminator. There was no clear correlation in the Circular Dichroism (CD) study of the different antiterminators. Enzymatic probing study showed that the loop region affected the A2 helix of the antiterminators but not the nature of the bulge.

Binding studies using fluorescence-based binding assays demonstrated that divalent metal requirement for tRNA binding to the antiterminator RNA is dependent on antiterminator sequence and structure. Monovalent metal ions alone did not sufficiently facilitate tRNA binding to the antiterminator. Interaction of small molecules with different antiterminators showed that the small molecules bind antiterminators differently due to RNA structural differences. Finally this study provided further evidence that binding of tRNA to antiterminator model occurs by means of an induced fit.

Approved: _____________________________________________________________

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1 Introduction

1.1 RNA structure and function

Ribonucleic Acid (RNA) is found in many organisms including plants, animals, bacteria and viruses (Lodish et al., 1995). There are five major types of RNA found in Eukaryotic cells. These include heterogeneous nuclear RNA (hnRNA), mRNA, tRNA, rRNA, and snRNA. Structurally, hnRNA and mRNA are both single stranded, while rRNA and tRNA form three–dimensional molecular configurations. Each type of RNA has a different role in various cellular processes. RNA functions differs from Deoxyribonucleic Acid (DNA) in that DNA stores genetic material while RNA functions as a carrier of genetic information, an adapter molecule in protein synthesis and a structural molecule in cellular organelle (Caprara and Nilsen, 2000). It has also been implicated to play a role in regulation of gene expression and a catalyst of biochemical reactions (Fedor and Williamson, 2005) that was thought to be only reserved for proteins. In addition to the above functions, RNA plays an important role in particular viruses to cause infections (Mu et al., 2007).

Chemically, RNA differs from DNA by the presence of an OH at 2′ of the ribose sugar (Figure 1.1). The consequence of the OH group renders RNA more susceptible to hydrolysis (Mikkola et al., 1999) and more flexible than DNA and preferentially forms A-form duplex conformations in RNA (Bloomfield et al., 2000). Stabilization of an RNA molecule is achieved by hydrogen bonding, stacking interactions, protonated base pairs, and the overall 3D structure (Bloomfield et al., 2000).

The base pairing patterns of RNA differs from DNA. In the case of a DNA molecule, the hydrogen bonding between the bases takes place through the formation of standard Watson-Crick (WC) base pairs. In the case of RNA apart from the standard WC
base pairs, the hydrogen bonding between bases can occur through highly versatile non-WC base pairing patterns often called non-canonical base pairs (Figure 1.2) (Blackburn et al., 2006). The most common non-canonical base pairs include the G.U wobble pair, the Hoogsteen, the A°C pair and G.A pair (Blackburn et al., 2006; Leontis et al., 2002). Generally, an RNA molecule is more stable in A-form geometry in which the furanose ring is in 3′-endo pucker and the glycosidic bond is in anti conformation (Cheatham and Kollman, 1997).
Figure 1.1 An example of an RNA strand connected by phosphate linkage. Phosphate groups connect the nucleotides at the 5′ of the ribose ring. The presence of the 2′ OH group in RNA renders it susceptible to hydrolysis (Blackburn et al., 2006).
Figure 1.2 Base pairing patterns in RNA (Blackburn et al. 2006). The G.C and A.U pairs represent the standard Watson and Crick pairs while G.U represents the non-canonical wobble.
1.2 The hierarchical nature of RNA

The RNA molecule is hierarchical in structure (Brion and Westhof, 1997) in which the higher orders assemble from preformed primary or secondary structures. The primary sequence determines the secondary structure, which in turn determines its tertiary formation.

The RNA primary structure is comprised of nucleotides written as textual strings using letters A, C, G and U to denote Adenine, Cytosine, Guanine and Uracil bases respectively. The sequence of the nucleotides depends on a particular RNA molecule and is written from the 5′ to 3′ direction.

The secondary structure is described by a list of canonical base pairs formed from the primary sequence and intervened by unpaired regions that form hairpin loops, bulges and internal or multiple loops (Wyatt et al., 1989). Another feature of secondary structure is stacking of the bases to the adjacent bases to form helical structures. Examples of RNA secondary structural elements include single stranded regions, duplexes, hairpin loops, junctions and bulges (Batey et al., 1999) (Figure 1.3). Single stranded regions consist of unpaired nucleotides at the 5′ or 3′ end of an RNA molecule, or between duplex regions of an RNA secondary structure. Regions containing single strands are roughly ordered by base stacking in a helical geometry. Hairpins consist of a duplex bridged by a loop of unpaired nucleotides. They are formed when an RNA strand folds back to form a base pair. The smallest loop is 2-3 nucleotides, but loops containing 4-5 nucleotides are the most stable (Groebbe and Uhlenbeck, 1988). Bulges have one or more unpaired nucleotides on one strand only, and the bulge can either stack into or out of the strand depending on the sequence context (Wyatt et al., 1989). The bulged nucleotides in RNA may cause the molecule
to bend (Bhattacharyya et al., 1990). The degree of bending depends on the number of bulged bases.

The tertiary structure is its three dimensional structure. Although RNA molecules are commonly represented as two-dimensional structures, most RNAs are folded into compact defined tertiary structures, necessary for their function (Golden et al., 1998; Latham and Cech, 1989). The formation of tertiary interactions depends on the secondary structure. Most tertiary interactions involve non-canonical base pairing or backbone-backbone interactions. Specific tertiary structures are seen in transfer RNA (tRNA), ribosomal RNA (rRNA), small nuclear (snRNA), certain introns and ribozymes (Batey et al., 1999). Even though mRNA is mostly known to function at primary the structural level, it can also adopt some complex tertiary structures, especially in the untranslated terminal regions (Nahvi et al., 2002). These can be essential for proper gene expression. Tertiary structural elements include pseudoknots, kissing hairpins, and triplexes (Moore, 1999),(Wyatt et al., 1990) (Figure 1.3).
Figure 1.3 Secondary and tertiary structural elements in RNA (Batey et al., 1999). A. Secondary structural elements are characterized by paired and unpaired bases and are typified by duplexes, hairpin loops and bulges. B. The tertiary structures formed from preformed secondary structures include kissing hairpins, pseudoknots and two-stem junctions.
1.3 RNA folding

An RNA molecule is very flexible providing a basis for its functional diversity. This structural adaptability can lead to difficulties in defining a single native structure of RNA a phenomenon known as the “RNA folding problem” (Brion and Westhof, 1997; Sosnick and Pan, 2003). RNA readily folds back on itself thus adopting unique folds that provide its functional diversity. RNA folding is driven principally by hydrogen bonding and base stacking (Brion and Westhof, 1997). The folding is fulfilled with complex non-canonical base pairing interactions and highly dependent on environmental factors such as the presence of metal ions and protein cofactors (Herschlag, 1995; Misra and Draper, 1998; Shiman and Draper, 2000). Most RNA molecules go through a stringent folding process in which misfolded states are prevalent or are as stable as the native state (Pan et al., 1997). Thus it’s important to refold RNA by careful denaturation and renaturation procedures when handling RNA in vitro (Pan et al., 1997). In in vivo, the non-specific RNA proteins called chaperones are likely to assist in proper folding of RNA (Herschlag, 1995), by preventing misfolding or resolving misfolded structures. Another group of RNA binding proteins is thought to solve the problem of the thermodynamically unstable 3D structure by recognizing the correct tertiary structure and stabilizing it. A typical example of such proteins is shown by the CYT-18 and CBP-2 proteins in the 3D folding of group 1 introns (Caprara et al., 1996; Gampel and Cech, 1991; Garriga and Lambowitz, 1986; Guo and Lambowitz, 1992; Weeks and Cech, 1995).

1.3.1 Role of metal ions in RNA folding

RNA is polyanionic at physiological conditions (Westhof and Auffinger, 2000), therefore there is a tendency of strong repulsion from the negatively charged
phosphate backbone. An early event in RNA folding is the formation of the secondary structure. The formation of the secondary structure is facilitated by cations, especially the monovalent metal ions such as Na\(^+\) and K\(^+\) (Basu et al., 1998; Shiman and Draper, 2000). These are mostly required for stabilizing the negatively charged phosphate backbone that is drawn together during the folding process (Basu et al., 1998; Shiman and Draper, 2000). The tertiary structure formation involves the tight packing of RNA strands and the formation of cavities. This is facilitated by divalent metal ions particularly Mg\(^{2+}\) (Gonzalez and Tinoco, 2001; Tinoco and Bustamante, 1999).

Metal ions interact with RNA in a number of ways; first they can non-specifically screen the charge of the polyanionic backbone, thereby reducing the repulsion between RNA strands (Gonzalez and Tinoco, 2001). They can also bind to specific RNA sites, and without forming specific interaction provide local stability to regions of strongly negative electrostatic potential (Misra and Draper, 1998). In other ways they interact with RNA through outer-sphere contacts that are mediated by coordinated water molecule or they coordinate directly with RNA functional groups, particularly phosphate oxygens and base heteroatoms (Batey et al., 2000). Even though a variety of metal ions contribute to the RNA function, certain ones are strongly preferred.

Among the monovalent ions, K\(^+\) is the most preferred and often specifically required for folding of the secondary structure (Basu et al., 1998; Shiman and Draper, 2000). Other monovalent ions that contribute to folding of certain RNAs are Na\(^+\) and Li\(^+\) (Lorsch and Szostak, 1994). The most common metal ion required for RNA folding and catalysis is Mg\(^{2+}\) (Gonzalez and Tinoco, 2001; Pyle, 1996; Tinoco and Bustamante, 1999). In some rare cases Ca\(^{2+}\), Mn\(^{2+}\), and Cd\(^{2+}\) can substitute for Mg\(^{2+}\). Trivalent ions such as Tb\(^{3+}\) and Co(NH\(_3\))\(_6^{3+}\) can be useful as structural probes of Mg\(^{2+}\) even though they are not natural co-factors of RNA. Tb\(^{3+}\) has been used as a Mg\(^{2+}\) binding probe in
the hammerhead ribozyme (Feig et al., 1999) while Co(NH$_3$)$_6^{3+}$ has also been a valuable probe for evaluating metal ion binding through outer-sphere mechanisms (Gonzalez and Tinoco, 2001; Kieft et al., 1999). Multivalent ions stabilize the RNA more effectively than the monovalent ions. For example the folding of the *Tetrahymena* group 1 ribozyme requires µM trivalent cations, mM divalent ions and M monovalent ions (Heilman-Miller et al., 2001). The size of the metal ion also affects the folding of RNA. For instance small metal ions such as Mg$^{2+}$ have been shown to stabilize more than larger ions such as Ba$^{2+}$ (Heilman-Miller et al., 2001). The same trend was also observed with polyamines (Koculi et al., 2004). The small ions tend to pack more closely around the RNA than the large ions thus stabilizing it better than the later (Rouzina and Bloomfield, 1996).

### 1.3.2 Role of a protonated base in RNA folding

The role of monovalent and divalent metal ions has been studied in depth. However little is known about the role of protons. Protonated nucleobases have the potential to play functional roles in both catalysis and folding (Cai and Tinoco, 1996; Legault and Pardi, 1997). Nucleobases with shifted pKa values may assist in the folding of certain RNA structures (Nixon and Giedroc, 2000). For example, the protonation of cytosine drives pseudoknot formation via a triplex (Nixon and Giedroc, 2000). A protonated base pair has also been shown to participate in rRNA structural interactions (Kubarenko et al., 2001). Furthermore a non Watson-Crick base pair CH$^+$-U that existed at pH 5.5 but was rearranged at higher pH (7.5) has been discovered in the structure of an oligonucleotide analog of ‘A loop’ region of *E.coli* 23S rRNA (Blanchard and Puglisi, 2001). It has also been reported that the internal loops in the 5’ untranslated region of the Turnip yellow mosaic virus RNA that serves as
encapsidation initiation signal are stabilized by the C.C and C.A mismatches (Bink et al., 2002). These mismatches rely on their pH-dependent protonation. Encapsidation was severely affected by mutants lacking protonatable bases. The NMR and UV melting studies confirmed that the C.C and C.A mismatches stabilized the structure when the pH is lowered from 7 to 5 (Hellendoorn et al., 1996; Hellendoorn et al., 1997). Elsewhere, it has been reported that a pH sensitive RNA tertiary interactions affects self-cleavage activity of the Hepatitis Delta Virus (HDV) ribozyme in the absence of added divalent metal ion (Wadkins 2001). The HDV ribozyme requires a mM concentration of divalent metal ions for optimal activity (Kuo et al., 1988; Sharmeen et al., 1988; Wu and Tinoco, 1998). However, at low pH, a form of the ribozyme will self cleave in high concentrations of monovalent salt without addition of magnesium (Nakano et al., 2000).

### 1.3.3 Role of loops in RNA folding

Loops are single stranded regions found in RNA secondary and tertiary structures. The loops form RNA hairpins representing structural fragments. These structural elements allow RNA to fold back onto itself forming tertiary structures necessary for stabilizing the RNA (Uhlenbeck, 1990). They play important roles in RNA structure and functions, for example hairpins are thought to provide nucleation sites for RNA folding (Uhlenbeck, 1990) and tertiary recognition sites for both proteins and nucleic acids (Jagath et al., 2001; Legault et al., 1998).

A majority of loops include the tetraloops, comprised of four ribonucleotides that form the most recurring motifs observed in natural RNAs (Tuerk et al., 1988b). About 70% of these tetraloops belong to the GNRA and UNCG families, Where N = U, A, C or G, R=G or A (Uhlenbeck, 1990; Woese et al., 1990). These families of tetraloops
have been shown to be unusually stable (Antao et al., 1991; Antao and Tinoco, 1992; Dale et al., 2000) provided that their stems consists of at least two G.C base pairs (Antao et al., 1991; Antao and Tinoco, 1992; Molinaro and Tinoco, 1995; Santalucia et al., 1992). In particular tetraloops containing sequences GAAA and UUCG form unusually stable hairpins and they have also shown to play some important biological functions. The UUCG is the terminal site of reverse transcriptase (Tuerk et al., 1988b) while the GAAA participates in the long-range tertiary interactions in catalytic RNAs (Pley et al., 1994). Also the GNRA tetraloops have been shown to participate in tertiary interactions that contribute to the formation of the proper three-dimensional structure of many RNAs (Moore, 1999; Varani, 1995). For example, interactions between GNRA tetraloops and RNA structures called tetraloop receptors have been observed in structures of Group I introns, the hammerhead ribozyme, and the ribosome (Cate et al., 1996; Nissen et al., 2001; Scott et al., 1995; Wimberly et al., 2000). These interactions have been demonstrated to contribute to stability and activity of folded RNAs.

The stability of these tetraloops arises from the conformation of nucleotides in the hairpin loop. The first and the fourth nucleotides of the hairpin loop of the GNRA tetraloop form a noncanonical G-A base pair, while nucleotides two to four form stacking interactions. In addition, there are several 2’OH-base hydrogen bonds (Sorin et al., 2002; Williams and Hall, 2000).

### 1.4 RNA regulation of gene expression

The structure of RNA has been demonstrated to play a crucial role in gene regulation of various systems (Mironov et al., 2002; Nahvi et al., 2002; Winkler et al., 2002). Sometimes these structures require interaction with proteins to carry out regulation or sometimes it is the RNA structure itself that directly affects regulation.
RNA was first discovered to regulate gene expression in the trp operon of *Escherichia coli* (Jackson and Yanofsky, 1973). This operon is regulated in response to the tRNA charging ratios (Oxender et al., 1979) by ribosome mediated-transcription termination. The leader region preceding the antiterminator contains a fourteen residue-coding region (trpL) which includes two tandem tryptophan codons. The binding of charged tRNA\textsuperscript{Trp} at two tandem Trp codons upstream of *trp* genes is required to facilitate translation of the peptide. In bacteria, since transcription is coupled with translation, a deficiency in charged tRNA\textsuperscript{Trp} causes the stalling of the ribosome at one of the two tandems Trp codons. This allows the antiterminator to form preventing the formation of the terminator leading to read through and transcription of downstream coding regions. When there is sufficient tryptophan, the ribosome translating the trp leader does not stall at the tandem Trp codons in trp leader, the trp leader stop codon is reached. The terminator forms, resulting in transcription termination.

Another example in which RNA is involved in gene regulation is through protein interactions with the RNA (Mironov et al., 2002). Some proteins can bind to specific RNA sequences thus enhancing repression of transcription termination. These proteins include the trp RNA-binding Attenuation Protein (TRAP), involved in the regulation of the *B.subtilis* trp operon (Mironov et al., 2002).

### 1.4.1 The T Box transcription antitermination mechanism

#### 1.4.1.1 Regulation

Expressions of many genes in bacteria such as those encoding the amino acid biosynthesis and aminoacyl tRNA synthetases are induced when the levels of cognate amino acid decrease (Grundy and Henkin, 1993; Henkin, 2000). In many Gram–positive bacteria, these genes are regulated by a common transcription antitermination
mechanism (Henkin et al., 1992). Comparative analysis has identified 805 genes in Gram-positive bacteria that contain a translated region of conserved 14-nucleotide (AGGGUGGNACCGCG) sequence known as the T Box, in the upstream of the transcription terminator site (Vitreschak et al., 2008). The genes that contain this sequence are classified as T box genes. Among the bacterial genomes that exhibit this system include the genera of Bacillus, Carboxythermus, Clostridium, Deinococcus, Enterococcus, Geobacter, Lactobacillus, Corynebacterium, Listeria, Lactococcus, Streptomyces Symbiobacteriu, Mycobacterium, Staphylococcus and Streptomyces (Grundy and Henkin, 1993; 1994a; Grundy et al., 1997; Henkin, 1994; Putzer et al., 1995).

In addition to the T box sequence, these genes consist of other conserved features both at the primary and secondary level. These features include, stem loop I containing a codon-like sequence in a specific region designated the specifier sequence and two mutually exclusive terminator and the antiterminator structures (Grundy and Henkin, 1994a; Henkin et al., 1992). The formation of the terminator leads to transcription termination. The formation of the antiterminator leads to read-through and full synthesis of the gene. Other conserved features have also been identified within T box genes. These include the GA motif at the base of stem I, the AGAGA sequence, the AG box in the bulge of within stem I, the GAAC box and the F box (Grundy and Henkin, 1994a; Henkin, 1994; Rollins et al., 1997). Studies on the Bacillus subtilis tyrosyl tRNA synthetase gene (tyrS) gene indicated that most of these conserved features are required for antitermination (Rollins et al., 1997; Winkler et al., 2001).
1.4.2 Mechanism

Uncharged tRNA acts as the effector for transcription antitermination of the T box family of genes. A reduction in the charging ratio of a specific tRNA class leads to differential regulation of genes in this family. For example, **tyrS** genes respond specifically to uncharged tRNA\(^{\text{Tyr}}\) (Henkin et al., 1992), and **thrS** and **thrZ** genes responded to uncharged tRNA\(^{\text{Thr}}\) (Putzer et al., 1992).

For antitermination to occur, the uncharged tRNA has to interact with the mRNA leader region in at least two positions (Figure 1.4). One of these positions involves the base pairing of the anticodon loop with the conserved specifier sequence in the leader region (Grundy and Henkin, 1993; Grundy et al., 1997). The second interaction forms between the acceptor end of the tRNA (5’-NCCA3’) and the first four nucleotides (5’-UGGN3’) of the antiterminator bulge (Grundy et al., 1994b). The variable base N of the bulge co-varies with the discriminator base N of the tRNA (Grundy et al., 1994b).

The interaction of charged tRNA with the mRNA leader region through anticodon-codon base pairing with the specifier sequence leads to formation of the terminator element (Figure 1.5). However it is presumed that the presence of an attached amino acid prevents the (-NCCA3’) sequence of tRNA from base pairing with the nucleotides of the bulge due to steric hindrance (Grundy et al., 2005). The absence of this second interaction favors the formation of the terminator stem loop leading to transcription termination. It is predicted that the terminator structure is more stable than the antiterminator with a \(\Delta G\) of -18 kcal versus -6.4 kcal (Mathews et al., 1999). But once the antiterminator structure is formed, it prevents the formation of the terminator by sequestering part of the terminator sequence (Grundy and Henkin, 1993).
Regulation of T Box genes was demonstrated to involve RNA-RNA interaction without a ribosome or co-factors in the *Bacillus subtilis* glycine synthetase (glyQS) leader (Grundy et al., 2002). This study showed that the uncharged tRNA induced antitermination with similar specificity both *in vivo* and *in vitro*. But such attempts failed to be demonstrated in the tRNA<sup>Tyr</sup>-directed antitermination of the *B.subtilis tyrS* leader (Grundy et al., 2002) or tRNA<sup>Thr</sup>-directed antitermination of the *B.subtilis thrS* gene (Luo et al., 1998).

The leader RNA-tRNA interaction is complex and requires multiple contacts (Grundy et al., 1997). Attempts to force interaction by *tyrS* leader with non-cognate tRNAs by alterations in known leader RNA specificity determinants indicated that some tRNAs can interact fairly efficiently with the *tyrS* leader to promote antitermination while others do not (Grundy et al., 1997).
Figure 1.4 The T box antitermination mechanism found in many Gram-positive bacteria. The antitermination is formed due to high ratio of uncharged to charged tRNA. The uncharged tRNA interacts with the specifier sequence through codon anti-codon base pairing and by through the acceptor end of the tRNA and the first four nucleotides of the bulge. The antiterminator structure forms and RNA polymerase continues to transcribe the upstream genes. Adapted from (Henkin and Yanofsky, 2002).
Figure 1.5 The T box termination mechanism found in many Gram-positive bacteria. The terminator forms as a result of high ratio of charged to uncharged tRNA. The charged tRNA interacts with the specifier sequence through codon anti-codon base pairing but the presence of an amino acid hinders the second base pairing. A terminator stem loop forms leading to transcription termination. Adapted from (Henkin and Yanofsky, 2002).
Figure 1.6 Interactions between uncharged tRNA and the leader region. The uncharged tRNA interacts with the leader region in at least two positions: The first pairing involves the anticodon of the tRNA and the specifier sequence while the second interaction that is critical for antitermination is between the acceptor end of the tRNA and the first four nucleotides of the bulge. Adapted from (Grundy et al., 2005).
1.4.3 T Box antiterminator element

The antiterminator element is made of two helices (A1 and A2) interrupted by a seven nucleotide bulge (Grundy et al., 2002a). For model RNA, see Figure 1.7. Phylogenetic and mutational studies on the A1 helix, A2 helix the bulge and the loop region of the antiterminator have shown some degree of conserved features in the T Box antiterminator element (Grundy et al., 2002a). While some highly conserved sequences within the helices can tolerate changes, others cannot. For example mutations in the highly conserved regions of the bulge and in the A2 helix has shown to reduce the function of the antiterminator, while mutations of other regions that were considered to be as high as 96% conserved did not have a major effect (Grundy et al., 2002a).

Mutations on the bulge region of antiterminator indicates that it has 7 nucleotides (Positions 6-12 in in antiterminator model AM1A or 219-225 in tyrS gene) in all antiterminators and has been proven to be essential for antitermination (Grundy et al., 2002a). This region was found to be 100 % conserved. Deletion of the bulge region resulted in a complete loss of transcription read through. The first four bases UGG of the antiterminator bulge are conserved in order to base pair with the CCA sequence universally found at the 3’ end of all tRNAs. A mutation in this region of the bulge abolishes read through (Grundy and Henkin, 1992). The fourth position of the bulge N varies with the discriminator position of the tRNA and base pairing between the tRNA and the bulge at this position is essential for read through (Grundy et al., 1994b). The positions 10-12 in the bulge that is (ACC) are conserved as A 100%, C, 87% and C,99% (Grundyetal.,2002a).
Figure 1.7 The T box antiterminator model RNA and tRNA models. The antiterminator AM1A represents 89% of all known leader regions, while its variant C11U represents 10% and has been shown to be less functional. Also shown is the tRNA-UCCA and microhelix UCCA/ACCA model tRNA. (Gerdeman et al., 2002).

Mutation of C at position 224 in \textit{tyrS} reduces antitermination. This can be compensated by increasing the uncharged tRNA concentration (Grundy et al., 2002a; Rollins et al., 1997).

Helix A1 was shown to vary in length, about 90% of the antiterminators contain 4-6 base pairs (Grundy et al., 2002a). Helix A2 also varies in length ranging from 3-12 bp. Most antiterminators contain helices with 4-7 nucleotides in length. The base pairs 14-23(C-G) and 15-22(G-C) in A2 helix are highly conserved with 96% and 99% conservation respectively (Grundy et al., 2002a). The loop region of the antiterminator was found to be considerably variable in sequence and length (Grundy et al., 2002a). Replacement of this AAUCA loop region with UUCG exhibited a normal expression and
induction in response to tRNA$_{\text{Tyr}}$ in $B.\ subtilis$ though the predicted stability for the mutant was shown to be higher (Grundy et al., 2002a). This suggests that the sequence in the loop is important in terms of an individual leader because of contextual effects or tertiary interactions with other regions of the leader (Grundy et al., 2002a).

1.4.4 T Box antiterminator model RNA and tRNA models

The T Box antiterminator model AM1A was constructed in order to facilitate \textit{in vitro} studies (Figure 1.7) (Gerdeman et al., 2002). The model based on $B.\ subtilis\ tyrS$ gene is found in about 89 % of known antiterminators and was found to be very functional (Grundy et al., 2002a). A model C11U with a substitution of a U at position 11 of the antiterminator model represents 10 % of known leader regions (Grundy et al., 2002a). This substitution conferred reduced expression of $B.\ subtilis\ tyrS$ gene (Grundy et al., 2002a). This model was also confirmed to bind tRNA models with lesser affinity (Gerdeman et al., 2002).

The NMR solution study indicates that the bulge region of the antiterminator model AM1A experiences some form of flexibility in the 5' end and stacking in the 3'end (Gerdeman et al., 2003). With the exception of U6 and C12, the bases in the bulge appear to be extruded from stacking on the flanking helical region. This exposes the hydrogen acceptor and donor groups of the bulge so that they can interact with other molecules. Bases A9-C12 form local stacking interactions; A9 is stacked to C25 and appears to be slightly accessible for binding to tRNA. A conformational rearrangement may occur as the other base of the bulge bind to tRNA allowing A9 to bind to tRNA as well. Thus the stacking in the 3' end may facilitate a set of conformations for the tRNA to sample during binding thus achieving an induced fit or tertiary structure capture (Gerdeman et al., 2003). A pyrimidine switch in C11U
perturbs this stacking making it less functional (Gerdeman et al., 2002; Rollins et al., 1997). An NMR study also indicates that there is a pronounced kink about 80° between helix A1 and A2 due to interruption by the 7-nucleotide bulge (Gerdeman et al., 2003). Helix A2 in the solution structure is characterized by a widening of the RNA helix groove at G13:U24 and C14: G23. The deformation is most likely as a result of accommodating the strain induced by the immediately adjacent extensive stacking within the bulge (Gerdeman et al., 2003).

A variant of tRNA^{Tyr} was constructed to ensure complete complementarities between the discriminator base with the appropriate variable base in the bulge of antiterminator AM1A (Gerdeman et al., 2002). This tRNA, designated tRNA-UCCA, has a U in position A73 of the wild type tRNA (Figure 1.7). Expression of this tRNA in vivo resulted in efficient antitermination of a matching tyrS leader (Grundy and Henkin, 1994a). Microhelix tRNA models mh-UCCA and mh-ACCA (Figure 1.7) were also constructed to investigate other features of tRNA structure required for binding specificity and effect of the discriminator base (Gerdeman et al., 2002).

1.4.5 In vitro structure-function studies of T box antiterminator

A number of in vitro studies based on the B. subtilis antiterminator RNA have shown that the discriminator position in tRNA plays an important role in the specificity and affinity of antiterminator RNA-tRNA interaction (Gerdeman et al., 2002; Means et al., 2007a). Gel mobility shift assays indicated a lack of an observed shift with a single mismatch in the acceptor end of tRNA when three base pairing interactions were still maintained. Steady state fluorescence binding assays have also shown a similar correlation, whereby; tRNA-UCCA bound AM1A with fivefold higher affinity than the mismatched model tRNA-ACCA (Means et al., 2007a).
Apart from the acceptor end of the tRNA, *in vitro* studies have confirmed that other features of the tRNA are essential for binding the antiterminator bulge (Gerdeman et al., 2002; Means et al., 2007a). According to these studies, full tRNA bound the antiterminator AM1A more strongly than the microhelix-UCCA, the minihelix or the tetramer (other model tRNAs). From the gel shift studies, full tRNA bound antiterminator AM1A with a $K_d$ of 63 µM as compared to a $K_d$ of 830 µM of the microhelix-UCCA. A similar trend was also observed by fluorescence based binding studies (Means et al., 2007a). These findings were reinforced by the tRNA randomized selection studies, in which only those tRNA that retained significant elements of tRNA tertiary structure were able to bind the antiterminator RNA, implying that structural features of the tRNA beyond acceptor end are essential for binding to the antiterminator (Fauzi et al., 2005). In addition, there is also evidence that the binding of full tRNA and microhelices to a variant antiterminator model C11U is weaker than that of AM1A, implying that C11U is a reduced-function model.

### 1.4.6 Role of Mg$^{2+}$ in tRNA binding and antitermination

The dependence of antitermination on tRNA binding was shown to be Mg$^{2+}$ dependent in the *in vitro* T box transcription antitermination assay (Grundy et al., 2002a). It was also demonstrated that Mg$^{2+}$ was required for proper folding of the glyQS leader RNA folding and its interaction with tRNA$^{\text{Gly}}$ (Yousef et al., 2005). A concentration of 5 mM Mg$^{2+}$ was sufficient for proper folding of the leader RNA and tRNA$^{\text{Gly}}$ binding but tRNA$^{\text{Gly}}$-directed antitermination required up to 15 mM Mg$^{2+}$ (Yousef et al., 2005). This study showed that there was no detection of binding of tRNA$^{\text{Gly}}$ to the glyQS leader RNA at 0 mM Mg$^{2+}$. Partial binding was observed between 2 and 4 mM while near maximum binding was observed at 5 mM or 15 mM of Mg$^{2+}$.
It has also been observed that Mg\(^{2+}\) is required for facilitation of binding of the tRNA to the antiterminator AM1A but not for pre-organization of the bulge (Jack et al., 2008). The investigation of the binding sites of Mg\(^{2+}\) in the antiterminator AM1A and C11U indicated a low affinity, hydrated metal ion binding in the A1 helix of the functionally relevant antiterminator AM1A but none was observed in the less functional antiterminator C11U. This type of binding was also observed in the G-rich region of microhelix-UCCA (Jack, 2007). The fluorescence and NMR studies also provided evidence that a threshold of Mg\(^{2+}\) is required for binding of the tRNA to the antiterminator model (Means, 2007b). NMR studies also point out that the nature of the bulge does not significantly change in the presence of Mg\(^{2+}\).

1.5 RNA as drug target

The complex function of RNA molecules in control of gene expression, through transcription regulation, translation regulation and protein synthesis (Manche et al., 1992; Moore and Steitz, 2002) provides numerous opportunities to target specific RNA structures for treating a variety of diseases. Its functional diversity can be attributed to the intricate three-dimensional folds it can assume through multitude of secondary and tertiary interactions (Chastain and Tinoco, 1991). Given these diverse functions, small molecules that selectively bind to RNA may provide intriguing points of therapeutic intervention. So far there are three classes of small molecule target classes: antibacterial, antiviral and mRNA (Thomas and Hergenrother, 2008).

1.5.1 Known RNA ligands

The discovery of several classes of antibiotics among them aminoglycosides to interact with rRNA triggered an interest in RNA-ligand recognition (Moazed and Noller,
Since then, aminoglycosides have been shown to interact with a number of functional RNA molecules such as group I introns (Vonahsen and Schroeder, 1991), hammerhead ribozyme (Stage et al., 1995) and the HDV ribozyme (Rogers et al., 1996). Also aminoglycosides were the first class of structurally diverse molecules discovered to disrupt the Trans-acting region (TAR) RNA responsible for gene regulation in Human Immunodeficiency Virus type 1 (HIV-1) (Mei et al., 1998; Mei et al., 1997; Mei et al., 1995).

More recently, other classes of small molecules have been found that selectively inhibit this system. Such molecules include polyamine-acridine-based compounds (Gelus et al., 1999; Hamy et al., 1998). The 2,4-diaminoquinoxalines and quinoxaline -2,3-diones have also been shown to selectively and stoichiometrically bind TAR in nanomolar range (Mei et al., 1998). The most recent antibiotics are the oxazolidinones with linezoid representing the first class to reach clinical level (Moellering, 2003). It has been demonstrated that oxazolidinones inhibit protein synthesis by binding the P site of 50s ribosomal sub-unit thus preventing the initiator tRNA from binding (Aoki et al., 2002; Chow and Bogdan, 1997).

Most clinically approved antibacterials target the rRNA by binding to the A site and preventing protein synthesis (Carter et al., 2000; Francois et al., 2005; Shandrick et al., 2004). Other antibacterials include those that target the amino-acyl tRNA synthetases and tRNA. A number of aminoglycosides have been shown to bind to tRNA\textsuperscript{Phe}. The ability of aminoglycosides to bind the T box antiterminator was demonstrated using FRET studies (Means and Hines, 2005). This study indicated that the binding of aminoglycosides to the antiterminator model RNA was in part through electrostatic interactions that are similar to how the aminoglycosides bind other RNAs. Among the eight different aminoglycosides tested, neomycin B had the lowest
dissociation constant. The trend for the dissociation constant correlated with the number of amines present.

The indication by FRET that neomycin B altered the bend angle between helix A1 and helix A2 of the antiterminator also implied that the binding site of aminoglycosides was in the bulge region (Means and Hines, 2005). Further studies showed that neomycin B enhanced RNase T1 cleavage at G15 of the antiterminator model AM1A implying that binding in the bulge region leads to a conformational change in the A2 helix (Anupam et al., 2008). Neomycin B was also demonstrated to displace monovalent metal ions upon binding to the antiterminator model (Anupam et al., 2008).

However, neomycin B did not significantly change the affinity of tRNA binding to the antiterminator. This led to a conclusion that electrostatic attraction alone is not sufficient to disrupt tRNA binding to the antiterminator (Anupam et al., 2008). A better way hypothesized was to use non-ionic compounds. Compounds such as oxazolidinones are a class of less highly charged compounds that have been found to bind RNA (Aoki et al., 2002; Colca et al., 2003; Matassova et al., 1999). A small library of 3,4,5-trisubstituted Oxazolidinones was synthesized and evaluated for their ability to bind the T box antiterminator RNA AM1A or the less functional C11U construct (Means et al., 2006). The binding of these compounds to the T box antiterminators was monitored using the 2-aminopurine fluorophore in which 2-aminopurine base was substituted at position A9 of the antiterminators. In this investigation, it was observed that some compounds preferentially bound AM1A compared to C11U.
1.6 Gram-positive bacteria

Gram-positive bacteria are the bacteria, which retain the dark blue or violet stain (Jawetz et al., 2007). This group of bacteria is able to retain the stain due to the high amount of peptiglycan (a molecule made up of amino acids and sugar) in their cell wall (Madigan and Martinko, 2005). Their counterpart the Gram-negative lacks the peptiglycan and therefore requires a counter stain. There are many well-known bacteria Genera that are Gram-positive, such as *Bacillus*, *Listeria*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Clostridium*. The most common diseases caused by these organisms include, pneumonia, otitis media, sore throat, among others (Wilson et al., 2002). In order to cure such infections, antibiotic drugs have been used. However bacteria have developed resistance among the most commonly used antibiotic drugs and the resistance has spread (Levy, 1997; Neu, 1992; Normark and Normark, 2002). The resistance is not a recent phenomena as its believed to have started since the 1930s by the emergence of sulfonamide resistant *Streptococcus pyogenes* in military hospitals (Levy, 1982). Soon after this in 1940s, the Penicillin resistant *Staphylococcus aureus* attacked the London civilian hospitals very soon after the introduction of this drug (Berber, 1948). Thereafter, the *Mycobacterium tuberculosis* with resistance to streptomycin emerged in the community soon after the discovery of this antibiotic (Crofton and Mitchison, 1948).

There has also been multi drug resistance, which was first detected, in the late 1950s and the early 1960s among enteric bacteria such as *E. coli*, *Shigella* and *Salmonella* (Levy, 2001; Olarte and Perez, 1983). Since the 1980s, re-emergence of tuberculosis has occurred that is often multidrug resistant and enhanced by the Human immunodeficiency virus infection (Bloom and Murray, 1992).
The main targets of antibiotic drugs are bacterial protein biosynthesis, bacterial nucleic acid replication and repair and cell wall biosynthesis enzymes and substrates (Alberts et al., 2001). Bacteria have developed resistance in a number of mechanisms such as, inactivation of the antibiotic by modification of its active chemical moiety, specific modification of the macromolecular target by mutagenesis of key residues (Hiramatsu, 1998) and promotion of antibiotic efflux from the cell (Walsh, 2000).

1.7 Significance of this research

As a result of the high bacterial resistance to antibiotics, there is a need to manage and prevent the drug resistance. A number of ways can be employed such as tracking the resistance frequency, isolating hospitalized individuals with potentially dangerous resistant bacteria and introducing new therapeutic approaches (Doern et al., 1999; Turnidge et al., 1989). The development of new drugs that can either block or circumvent resistance mechanisms or those that attack new targets is essential. One good possibility is targeting the T Box antitermination mechanism.

Further research is required in order to investigate how the tRNA actually binds and how small molecules can disrupt the binding of the tRNA. This addresses some factors that affect the structure and function of the antiterminator element. The advantages of targeting this system are that many genes are regulated by the T box antitermination mechanism due to response to a cognate uncharged tRNA. Also specific RNA sequences and structures are involved in mediating of crucial regulatory decisions. This can be exploited to allow premature transcription termination in response to a specific physiological signal. By targeting this system, the resistance mechanism of bacteria through mutagenesis will be reduced due to the difficulty of the bacterial system to mutate all the genes involved. This research provides detailed
structural and functional information to determine the influence of some antiterminator features thus to help determine the most relevant antiterminator and tRNA models for small molecule screening.

Hypothesis I
The loop region closing antiterminator helix A2 or the microhelix could have an effect on the structure and function of the antiterminator.

In previous enzymatic studies, it was observed that the A2 helix of the antiterminator AM1A became less ordered and therefore a less A form geometry upon binding of neomycin B or tRNA (Anupam, 2007). Likewise the tRNA acceptor stem became more flexible upon binding to the antiterminator (Agyeman, 2007). Based on these results, it was hypothesized that the loop region had an effect on both the antiterminator structure, and the microhelix structure, which could affect the binding affinity of tRNA or small molecules.

Hypothesis II
The divalent metal ion requirement for tRNA binding to antiterminator RNA is dependent on antiterminator sequence and structure.

Magnesium chloride was shown to bind diffusely in the G rich region of the A1 helix of the antiterminator AM1A (Jack, 2007). It was then hypothesized that the binding of tRNA to antiterminator is facilitated by the divalent metal ions especially Mg$^{2+}$ and that monovalent metal ions alone are not sufficient to facilitate the binding. In addition, the effect of Mg$^{2+}$ on the binding is hypothesized to be dependent on the antiterminator sequence and structure.

Hypothesis III
Different antiterminators will bind small molecules with differing specificity due to RNA structural differences.
1.8 Review of methods

1.8.1 MFold thermodynamic stability

This is a method of predicting the RNA secondary structure from its primary sequence through energy minimization (Zuker and Sankoff, 1984). Free energies are calculated from experimentally determined parameters using nearest-neighbor models for regions, which form base pairs (Borer 1974). MFold determines the optimal (Minimum energy) structure and a set of suboptimal foldings that are within the users default settings of the minimum energy structure.

As a result of uncertainties in the thermodynamic data (Zuker, 1989b), the set of suboptimal foldings exists and covers such a large area of structural space. The parameters used to control RNA secondary structure prediction by MFold are window size (W), percent sub-optimality (P) and the inclusion or exclusion of additional energy calculations based on coaxial stacking (Freier et al., 1986). MFold has some constrains in that base triples are not included in the secondary structures; hairpin loops with less than two bases and pseudoknots are prohibited and only G.C, A.U, and G.U base pairs are formed (Zuker et al., 1999). This approach can only be considered as crude since its not easy to quantify energies for loop regions because the free energies was simply assumed to depend on the number of nucleotides in the loop, thus using only the entropic contribution to the free energy of formation. There are loops like the tetraloops whose energetics strongly depend on the sequence due to the formation of non-canonical pairing (Jucker et al., 1996).
1.8.2 UV monitored thermal denaturation (UV-absorbance melting curves)

The folding and stability of nucleic acids can be determined by UV absorbance melting curves (Puglisi and Tinoco, 1989; SantaLucia and Turner, 1997). Such experiments can be used to determine the stability of the secondary structure of DNA or RNA. It may also be used to study the hybridization between a target and an oligonucleotide, which may be used for PCR primer design, sequencing or hybridization (Owczarzy et al., 1997).

The UV absorbance is an optical spectroscopy that can monitor the nucleic acid transition between the native and denatured or single stranded state induced by temperature (Blackburn et al., 2006). As the temperature increases an RNA molecule looses its native nature and the ratio of molecules in the native versus denatured decreases. A transition between these two states can be determined by change in absorbance with temperature. Since the denatured and the native have different molar absorptivities. The mid-point of the transition is called the melting temperature \( T_m \). The UV absorbance-melting curve shows an increase in absorption as stacked bases in the native conformation become unstacked in the denatured state. This increase in absorption is called hyperchromicity (Tinoco 1960) and the decreased absorption is called hypochromicity (Cantor and Schimmel, 1980a).

1.8.3 Circular dichroism (CD)

The CD is a form of spectroscopy based on the differential absorption of left- and right-handed circularly polarized light, which arise due to structural asymmetry (Johnson, 1996). The experimentally measured CD is the difference in absorbance for the left and right handed circularly polarized light, \( \Delta A = (A_L - A_R) \). By applying the Beer’s
law, this equation can be expressed as, \( \Delta A = (\varepsilon_L - \varepsilon_R)Cl \), where, \( \varepsilon_R \) and \( \varepsilon_L \) are molar extintion coefficient of right and left polarized light, \( C \) is molar concentration and \( l \) is the path leght in cm. CD is defined by \( \Delta \varepsilon \) that reflects the ellipticity of the molecule. The linear polarization of incident light in in an active medium can change to elliptical polarization. The ratio of minor to major axes of ellipse that is formed gives the tanget of ellipticity, \( \theta \). Most measurements in CD are reported in degrees of ellipticity (Johnson, 1996). The defination of this angle as a function of absorbance can be given by the following equation.

\[
\theta = 32.98 \Delta A
\]

The linear dependence of solute concentration and path length is removed by defining molar ellipticity as \([\theta]\) which is related to \( \Delta \varepsilon \) by the following equation (Johnson, 1996)

\[
[\theta] = 100 \theta / cl = 3298 \Delta \varepsilon.
\]

Whereby \( c \) = Concentration of RNA and \( l = \) the cuvette path length

When analyzing different RNA molecules, it is necessary to normalize the measured CD based on the number of nucleotides. This can be done by using the following equation for the molar circular dichroic absoption (Sosnick et al., 2000).

\[
\Delta \varepsilon \text{ (cm}^2 \text{ mmol}^{-1}) = \theta / 3298 \text{ x } C \text{ x } L \text{ x } N,
\]

Where \( C \) is RNA concentration, \( L \) is cuvette pathlength in cm and \( N \) is number of nucleotides in RNA.

The bases in nucleic acids have a plane of symmetry, thus are not intrinsically optically active (Tinoco et al., 1980). The CD in nucleic acids arises from the induction of the chromophonic bases by the asymmetric ribose and deoxyribose sugars (Specher and Johnson, 1977). The absorption of bases begins at around 300 nm. The electronic transition of the ether and hydroxyl group of the sugars begin at 200 nm, and
the electronic transitions of the phosphate groups begin further in the UV region (Johnson, 1996). The CD of nucleic acids is thus measured between 200 nm and 300 nm. The absorption of some buffers in the far UV region prevents the accurate measurements of CD below 200 nm.

CD is an ideal method for determining the secondary structure of proteins or nucleic acids in solution. Nucleic acids are polymorphic, that is, they can assume a variety of conformations depending on the salt, solvent and composition (Daly et al., 1990; Riazance et al., 1985). A normal conformation of RNA is A form at neutral buffer and moderate salt. The CD spectra of A-form RNA gives characteristic peaks with different properties, The 268 peak gives information about base pairing and stacking (Sosnick et al., 2000). The 208 nm peak indicates the orientation of the phosphate backbone, 278 nm peak decreases in the formation of tertiary structure. The 235-265 nm peak can reflect single stranded regions (Cox et al., 1976).

In polynucleotides, the hydrophobic stacking results in close contact and coulombic interactions that give rise to intense CD bands at 268 nm corresponding to each base transition (Fasman, 1996). The CD signal of nucleic acids increases with length due to the co-operativity of chiral interactions between continuous bases. This effect occurs both as a result of sequence effects arising from the nearest neighbor interactions as well as from the overall gross secondary structure (Gray et al., 2008).

The CD provides a quick, convenient and accurate picture of an overall conformation and secondary structure of a particular nucleic acid in solution and does not require large amounts of material (Kypr et al., 2009). It is an ideal method for determining the secondary structure of proteins or nucleic acids in solution.
1.8.4 Enzymatic probing

One important approach to study the structure of an RNA molecule in solution is the use of enzymatic and chemical probes (Knapp, 1989). Studies where enzymatic probes are used can generate preliminary detailed structural information on RNA molecules more readily than using optical, magnetic resonance or crystallographic techniques. Enzymatic probes can be used to provide information about the single and double stranded region of an RNA molecule (Cordier and Schon, 1999). RNase such as T2 and S1 cleave single stranded regions without specificity (Brunel and Romby, 2000). RNases T1 and Cl3 cleave the single stranded guanosines and cytosines respectively. RNase A cleaves single stranded cytosines and Uracils. The double stranded RNase V1 cleaves paired and stacked nucleotides without sequence specificity. While RNase V1 and S1 cleave 5’ phosphates, RNase A, T1 and Cl3 cleave the 3’ phosphates (Brunel and Romby, 2000; Qu et al., 1983). The single stranded specific S1 and the base pair specific V1 have been used to obtain information on the secondary structure of RNA molecules in solution such as tRNAs (Wurst and Vournakis, 1981), different sources of ribosomal RNAs (Troutt et al., 1982) and rabbit hemoglobin mRNAs (Vary and Vournakis, 1982). Partial kinetic condition digestion of the 5’ or 3’- end labeled RNA provides information on accessibility of the enzymes to phosphodiester bonds. This reveals their status as either single stranded or base-paired regions respectively at specific conditions of pH, ionic strength and temperature.

1.8.5 Fluorescence

Fluorescence is a luminescence that is mostly found as an optical phenomenon in which the molecular absorption of a photon triggers the emission of another photon with a longer wavelength. The energy difference between the absorbed and emitted
photons ends up as molecular vibrations or heat. Usually the absorbed photon is in the ultraviolet range, and the emitted light is in the visible range, but this depends on the absorbance curve and Stokes shift of the particular fluorescent substance (fluorophore) (Lakowiz, 1999). Fluorescence spectroscopy has been utilized to study the conformation of nucleic acids in solution (Klostermeier and Millar, 2001; Millar, 1996; Walter and Burke, 2000). Measurements of fluorescence can be classified into two groups; the steady state and the time resolved. The steady state measurements performed with constant illumination and observations while the time resolved is used to measure the intensity decays or anisotropy decays (Guest et al., 1991; Holzwarth, 1995). There are two main classes of fluorophores: the intrinsic and extrinsic fluorophores. Intrinsic fluorophores are those that occur naturally. They include the aromatic amino acids, NADH, flavins and derivatives of pyridoxal and chlorophyll (Levitt et al., 2006). These are mostly found in proteins. Extrinsic fluorophores are added to the molecule to provide fluorescence or to change the spectral properties of a molecule (Benson et al., 1993; Smith et al., 1986). Extrinsic fluorophores include dansyl chloride, fluorescein, rhodamine, and base analogs among others.

Based on the fact that unmodified bases A, C, G, and U of RNA have negligible fluorescence (Cantor and Schimmel, 1980b), fluorescence studies on RNA structure and dynamics requires synthesis of RNA with a chromophore connected via a linker or with a base analog such as 2-Aminopurine (2-AP) and isoxanthopterin (IXP) analogs for adenine and guanine respectively (Odom et al., 1988). Some of the fluorophores commonly used to study RNA include Fluorescein and Rhodamine B. The ability of fluorophores to reflect changes in their environment makes fluorescence a powerful tool in the study on nucleic acids conformation and dynamics. Furthermore
fluorescence techniques provide high detection sensitivity and can also be performed under a variety of solution conditions (Clegg et al., 1993).

Fluorescence resonance energy transfer (FRET) can be used to monitor the conformation of nucleic acids (Furey et al., 1998). The application of FRET requires for the nucleic acid to be synthesized with two fluorophores at two positions. One fluorophore acts as a donor while the other as an acceptor (Allen and Benkovic, 1989). Excitation of donor fluorophore energy is transferred to the acceptor by dipolar coupling of transition moments. The efficacy of energy transfer is strongly dependent on the distance between the donor and the acceptor. This effect can be exploited for structural analysis, such as the kink angle in bulged duplexes and the distances between helices in branched junctions (Allen and Benkovic, 1989; Clegg et al., 1992).
2 Results for structure-function studies of the antiterminator loop and variants

2.1 Determination of model RNA stability

2.1.1 Mfold thermodynamic stability

In order to investigate the effect of loop stability on the antiterminator structure and function, antiterminator models were designed with different loop substitutions. Loops are important regions of an RNA molecule because they are involved in the RNA folding mechanism. In this case they help in stabilizing an RNA molecule. The loops containing 4 nucleotides form a majority of all loops. These types of loops are classified under the GNRA and UNCG tetraloops that form about 70% of loops. These loops are also known to be very stable (Antao et al., 1991).

In the antiterminator model AM1A, the non-conserved loop region was replaced with the UUCG tetraloop (Gerdeman et al., 2002). In this investigation, the effect of the UUCG loop substitution was analyzed by studying different antiterminators with changes in the loop region. The UUCG tetraloop of antiterminator AM1A was replaced with various loops belonging to the GNRA and the UNCG tetraloops. These classes of tetraloops have been well studied. The resultant secondary structure was predicted using the Mfold thermodynamic stability server version 3.2. This method is used to give information about the secondary structure of an RNA molecule and its stability based on energy minimization (Zuker, 1989a; 2003; Zuker and Sankoff, 1984). Version 3.2 includes coaxial stacking. The method assumes that structure with the lowest energy readily forms and is the most stable. The Mfold version 3.2 was chosen over the newly implemented Zuker algorithms RNA structure version 4.6 because the latter
is only very useful if the RNA contains chemically modified nucleotides (Mathews et al., 2004).

Sequences that folded correctly with the lowest free energy ($\Delta G$) were selected. The antiterminators were grouped into two groups: those that were more stable than AM1A (AM3, AM4) and those that were less stable than AM1A (AM5, AM6). The closing loops in these antiterminators were UUCG for AM1A and C11U, GAAA for AM3, GCAA for AM4, UCCG for AM5 and GGGA for AM6. Figure 2.1. Shows the different antiterminator models folded by Mfold thermodynamic stability and their respective $\Delta G$ are shown in Table 2.1.

As shown in Table 2.1, AM3 and AM4 had the highest stability with a predicted $\Delta G$ of -13.9 kcal/mol while AM5 and AM6 had the lower stability with $\Delta G$ of -10.5 and -12.4 kcal/mol respectively. Also included in this study was the antiterminator AM2 with the original B. subtilis tyrS wild type loop sequence AAUCA and the GlyQS representing the B. subtilis glycine synthetase antiterminator with a GAAC tetraloop, this tetraloop does not belong to the GNRA or UNCG classes and is not listed among the known tetraloops. As shown in the table, both AM2 and GlyQS were predicted to be less stable than AM1A with $\Delta G$ value of -9.3 and -12.5 kcal/mol respectively. According to this data, the wild type AM2 was the least stable antiterminator.

Prediction of the RNA secondary structure using thermodynamic rules may sometimes be not very accurate because it is mostly based on certain rules, which are empirical. The actual stability might be different from the predicted. Due to the fact that this method is only based on certain parameters, it was necessary to determine the stability of these RNAs using another assay. The stability of these RNAs was then investigated by monitoring UV absorbance of thermal denaturation.
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Figure 2.1 Antiterminator model secondary structures with different substitutions in the loop region: AM2 has the \textit{B. subtilis} wild type loop, AM3, AM4 and AM6 have the GNRA and UNCG tetraloop while AM1A and AM5 have the UNCG tetraloop. GlyQS is the glycine synthetase antiterminator and its stability is less than AM1A. Also shown is the reduced function model C11U.

2.1.2 UV monitored thermal denaturation for determination of thermal stability

The UV absorbance monitoring during thermal denaturation was used to determine the stability of the different antiterminators based on their melting temperature ($T_m$). When nucleic acids are subjected to high temperatures, the base pairs are broken down and the molecule changes from its native (folded) to the denatured state (Blackburn et al., 2006). The mid point of this transition where half the molecule is still
folded and half is denatured is referred to as the melting point (Puglisi and Tinoco, 1989). The temperature at which it occurs is the $T_m$.

A more thermodynamically stable structure would require high temperature to disrupt the base pairs and thus the $T_m$ would be high. Base pairing and stacking affects the $T_m$ (Puglisi and Tinoco, 1989). Also the presence of metal ions such as Mg$^{2+}$ would raise the $T_m$ of an RNA molecule (Serra et al., 2002).

It was hypothesized that the antiterminators with the lowest predicted $\Delta G$ would be the most stable and thus have the highest $T_m$. The absorbance of 1.5 µM RNA of the different antiterminators was monitored at 260 nm between 4°C and 95°C in various salt and buffer conditions.

The first derivatives of the melt curves were determined using the OD Deriv program (Draper and Gluick, 1995) and plotted against temperature (°C). A representative of the plots is shown in Figure 2.2. The $T_m$ are shown in Table 2.1. As expected in low salt conditions, AM2 had the lowest melting temperature. Followed by GlyQS with the second lowest. Also as expected, AM3 had the highest melting temperature. The data correlates well with the MFold predicted thermostability. AM2 was predicted to be the least stable and AM3 the most stable. However AM4 and AM5 did not follow a correlation trend with predicted stability. The low $T_m$ for AM2 and GlyQS points clearly to the effects of the tetraloop on the stability of the antiterminator. This indicates that the loop region contributes significantly to the stability to the antiterminators. The AAUCA loop in AM2 and the GAAC loop in GlyQS are not characterized loops. Both the antiterminators had the lowest $T_m$ implying the lack of contribution of their closing loops to their stability.
The melts were also investigated in higher concentration of salts (50 mM Sodium phosphate pH 6.5, 50 mM NaCl, 5 mM MgCl₂ and 0.01 mM EDTA. In this study, the Tₘ of the antiterminators was raised by between 16 °C to 20 °C. Also AM2 had the lowest Tₘ followed by GlyQS. For the rest of the antiterminators, the trend correlated with the Mfold predicted thermostability apart from the antiterminator AM4 (Table 2.1). An attempt to use the same buffer conditions for higher concentration of Mg²⁺ (10 mM and 15 mM) failed because precipitation of the buffers occurred at higher temperatures during the melt cycles. In order to investigate the effects of metal ions on the folding of the antiterminators different conditions were chosen.

The melts were then analyzed in 10 mM sodium phosphate buffer pH 6.5, 90 mM NaCl, 0.01 mM EDTA and either no Mg²⁺ or with 5mM or 15 mM Mg²⁺. Such conditions had been demonstrated to be successful when investigating the effects of Mg²⁺ on stabilization of RNA oligomers of defined structures (Serra et al., 2002).

The melting temperatures of all the antiterminators increased significantly in the presence of NaCl as compared to when the metal ion was absent. In 10 mM sodium phosphate buffer and 90 mM NaCl, the change in Tₘ was about 13 °C and was almost similar in all the antiterminators apart from AM4, which had a change of 12 °C. When 5 mM Mg²⁺ was added, the Tₘ for most of the antiterminators was raised by about between 10.1 °C and 12.5 °C apart from antiterminator models AM4 and AM6 in which the Tₘ was raised by 14.4 °C and 13.7 °C respectively. At the highest concentration of Mg²⁺ (15 mM) the Tₘ for AM2 and AM4 was raised to a larger extend than the rest of the antiterminators. These antiterminator models showed an increase in Tₘ of 18.2 °C, 18.1 °C for AM2 and AM4 respectively.
The results from monitoring UV absorbance by thermal denaturation demonstrated a correlation with the predicted Mfold thermodynamic stabilities (Figure 2.3) even though there were a few exceptions. The monovalent metal ions stabilized all the RNAs in a similar manner except for AM4. But Mg$^{2+}$ had a greater effect on the models AM2 and AM4.

**Figure 2.2** A representative of UV derivative plots of antiterminator models with different loop substitutions showing the effect of Mg$^{2+}$ on melting temperature. AM1A, AM2, AM3, AM4, AM5, AM6, C11U and GlyQS. The thermal denaturation was monitored between 4 ºC – 95 ºC in 10 mM sodium phosphate buffer pH 6.5, 90 mM NaCl, 15 mM MgCl$_2$ and 0.01 mM EDTA.
### Table 2.1 The effect of loop stability and magnesium chloride on melting temperatures of different antiterminator RNA models

<table>
<thead>
<tr>
<th>Antiterminator</th>
<th>$\Delta G$ (kcal/mol) of desired structure</th>
<th>$\Delta G$ (kcal/mol) of close alternate structure</th>
<th>Melting Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>10 mM NPB pH 6.5, 0.01 mM EDTA</td>
</tr>
<tr>
<td>AM1A</td>
<td>-13.5</td>
<td>-11.7</td>
<td>53 ± 0.6</td>
</tr>
<tr>
<td>C11U</td>
<td>-13.5</td>
<td>-10</td>
<td>54 ± 0.3</td>
</tr>
<tr>
<td>AM2</td>
<td>-9.3</td>
<td>-5.8</td>
<td>42 ± 0.4</td>
</tr>
<tr>
<td>AM3 (GAAA)</td>
<td>-13.9</td>
<td>-12.1</td>
<td>54.1 ± 0.5</td>
</tr>
<tr>
<td>AM4 (GGAA)</td>
<td>-13.9</td>
<td>-12.1</td>
<td>51.2 ± 0.3</td>
</tr>
<tr>
<td>AM5 (UCCG)</td>
<td>-12.4</td>
<td>-10.6</td>
<td>54.2 ± 0.7</td>
</tr>
<tr>
<td>AM6 (GGGA)</td>
<td>-10.5</td>
<td>-9.3</td>
<td>51.9 ± 0.2</td>
</tr>
<tr>
<td>GlyQS</td>
<td>-12.5</td>
<td>-9.0</td>
<td>47 ± 0.5</td>
</tr>
</tbody>
</table>

*Values are average of duplicate measurements*

NPB is sodium phosphate buffer
Figure 2.3 The melting temperature versus Mfold predicted $\Delta G$ (kcal/mol) of different antiterminators at A, 0 mM Mg$^{2+}$, B, 5 mM Mg$^{2+}$ and C, 15 mM Mg$^{2+}$. 
2.2 Qualitative structural comparison

2.2.1 Circular dichroism (CD)

The CD was used to qualitatively compare the secondary structure characteristics of the different antiterminators. The CD data give information about the extent of base stacking, base pairing; single stranded regions and tertiary interactions (Johnson, 1996). The CD spectra of the antiterminators were obtained at 4 °C in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, and 0.01 mM EDTA and either 5 mM or 15 mM MgCl₂. The CD spectra are shown in Figure 2.4. The properties of different peaks in the CD were used to evaluate the conformation of the different antiterminator RNAs. The most useful peaks were the 268 nm and 209 nm peaks. The 268 nm peak gives information about the degree of base pairing and stacking (Sosnick et al., 2000). A peak of a higher intensity would imply a greater base pair formation or increased stacking (Sosnick et al., 2000). The 209 nm peak gives information about the conformation of the phosphate backbone. The higher intensity (more negative) would imply more stable conformation. In other words, an RNA molecule would be considered more stable if both the 268 nm and 209 nm peaks were proportionally intense.

The results show that the concentration of Mg²⁺ did not change the CD of the different antiterminators. The spectra for AM1A, AM2 and AM5 look similar in terms of the degree of stacking, or base pairing and in the phosphate backbone structure. All these antiterminators had a maximum ellipticity λ_max at 268 nm and a minimum at 209 nm. The antiterminator AM2 had a more intense λ_max at 268 nm and a more negative peak at 209 nm compared to AM1A and AM5. The other antiterminators AM3 and AM4 had a positive λ_max at 268 nm and 265 nm respectively at both concentrations of Mg²⁺ and a negative
Figure 2.4 The average of two CD spectra of antiterminator RNA models with different loop regions; AM1A, AM2, AM3, AM4, AM5, AM6, and GlyQS. The RNAs (10 μM) were scanned at 4 °C in 50 mM sodium phosphate buffer containing 50 mM NaCl, 0.01 mM EDTA 5 mM MgCl₂ spectra A and 15 mM MgCl₂ spectra B in a final volume of 200µl. This data is effectively normalized to account for the differences in the number of nucleotides.
\( \lambda_{\text{max}} \) at 208 nm and 209 nm respectively for 15 mM \( \text{Mg}^{2+} \). In contrast, the negative \( \lambda_{\text{max}} \) at 5 mM \( \text{Mg}^{2+} \) was 209 nm for both AM3 and AM4.

The antiterminator AM3 had a more negative \( \lambda_{\text{max}} \) at 5 mM \( \text{Mg}^{2+} \) than AM4. This trend was reversed at 15 mM \( \text{Mg}^{2+} \) whereby; AM4 had a more negative peak than AM3. GlyQS had the most intense positive \( \lambda_{\text{max}} \) at 268 nm, but the negative \( \lambda_{\text{max}} \) at 208 nm was of medium intensity compared to all the antiterminators. AM6 had the most negative \( \lambda_{\text{max}} \) at 209 nm and the least positive \( \lambda_{\text{max}} \) at 268 nm among all the antiterminators.

From the CD data, there were observed association in characteristics of the spectra of the different RNA models and their Mfold predicted thermostability. The most predicted stable structures had a very positive peak and a very negative peak (AM3 and AM4). The least stable had a less positive peak and a less negative peak. The antiterminators AM2, AM6 and GlyQS did not follow this trend.

Base pairing in RNA and other A-form characteristics increases the intensity of the \( \lambda_{\text{max}} \) 268 nM. The main difference between GlyQS and the rest of the antiterminator is because GlyQS has a total of 6 base pairs in the A2 helix while the rest have 4 base pairs apart from AM2, which has 5 base pairs (see Figure 2.1 for structure). This is likely to explain why the intensity of GlyQS at 268 nm is higher than the rest of the antiterminators.

### 2.2.2 Effects of pH on CD of AM1A and C11U

Base protonation has been demonstrated to play a functional role in folding and stabilization of certain structures like the pseudoknot formation (Nixon and Giedroc, 2000). An effect of pH on folding stabilization of the antiterminator was investigated using CD. The CD spectra of the antiterminator AM1A and C11U were obtained by
collecting scans at 4°C in the range of between 200 nm and 320 nm. The rest of the parameters were as described in the materials and methods. The scans were collected in different pH buffers. The different pH values investigated were pH 5.0, pH 5.5, pH 6.0, pH 6.5, pH 7.0, pH 7.5 and pH 8.0. The buffers used for these pH values were; Mes for pH 5.0, 5.5 and 6.0, sodium phosphate for pH 6.0, 6.5, 7.0 and Trizma for pH 7.5 and 8.0. These different buffers were chosen so as to ensure good buffering conditions. That is the pKa of the buffer chosen was close to the required pH. The rest of the procedure was followed as described in materials and methods.

The results show that the CD of AM1A was only slightly affected by the changes in pH and followed a particular trend (Figure 2.5). The CD of C11U did not follow a particular trend with changes in pH. The changes in AM1A were mostly observed in the \( \lambda_{\text{max}} \) 209 nm that is a characteristic of the stability in the phosphate backbone. The more negative it is the more A-form like geometry, while the less negative the less A-form geometry. The peak was more negative for pH 5.0 with a molar CD of \(-8.7 \text{ cm}^2\text{mmol}^{-1}\). This negative peak decreased in intensity as the pH increased to \(-7.6 \text{ cm}^2\text{mmol}^{-1}\) at pH 8.0. But for C11U there was no such trend both in the \( \lambda_{\text{max}} \) 209 nM and the \( \lambda_{\text{max}} \) 268 nM.

The CD studies only provided information about the secondary structure of the different RNA. Including the base pairing, the degree of stacking or the geometry. However, this assay could not provide information about the actual nucleotides involved in base pairing or stacking. This prompted the use of enzymatic probes to investigate the nucleotides involved in base pairing or stacking.
Figure 2.5 The analysis of a duplicate λ max 209 nm CD peak of AM1A and C11U at various pHs. The scans were measured at 4 °C in 50 mM buffers; Mes (pH, 5.0, 5.5, 6.0) Sodium phosphate (pH 6.5, 7.0) and Trizma (pH 7.5 8.0) containing 50 mM NaCl, 15 mM MgCl2 and 0.01 mM EDTA.

2.2.3 Enzymatic probing

To determine the secondary structure of the different antiterminators, enzymatic probing was employed. This method was chosen because it has flexible reaction conditions that can be conducted under physiological pH and can also provide information that could not be obtained from the spectroscopic methods. The single stranded specific RNase T1 and RNase A were employed. In order to get conclusive evidence for double stranded regions and exclude single stranded regions not cleaved due to steric hindrance, RNase V1 was employed. This is a non sequence specific RNase that cleaves the 5′ end of paired or stacked nucleotides (Brunel and Romby 2000). The RNAs were first labeled at the 5′ end using γ-P32 ATP. The rest of the
procedures of enzymatic probing can be found in section 5.9.1 of materials and methods.

### 2.2.3.1 RNase T1 probing

RNase T1 is a sequence specific RNase that primarily cleaves the 3’ side of unpaired Gs (Brunel and Romby, 2000). Figure 2.6 and 2.7 shows RNase T1 probing of the different antiterminators. The results show cleavage in helix A1 at nucleotides G1, A2 and A3 indicating single strand nature or flexibility in the region. The cleavage was similar in all the antiterminators. Following the cleavage in A1 helix, there was also a strong cleavage in the bulge region at G7 and G8 in all the antiterminators. This was expected because these nucleotides are involved in the base pairing with the tRNA. NMR solution study has also shown that there is flexibility in this region (Gerdeman 2003). The cleavage was higher in C11U compared to the rest of the antiterminators. The antiterminators AM1A, AM2 and AM3 followed with a much equal cleavage at G7 and G8. Then followed by AM4 and AM5 then AM6. GlyQS was the least cleaved at both G7 and G8. The cleavage pattern in the bulge region also correlates with the Mfold thermodynamic stability data, by which, apart from AM4, the overall predicted stability correlated with increased cleavage at G7 and G8. High cleavage in these nucleotides indicates availability of the nucleotides to base pair with the tRNA or ligands.

Analysis of the A2 helix showed a clear difference in the cleavage pattern. The most significant cleavage pattern difference was the G13 and G15 of the A2 helix. In the antiterminators AM1A, AM4, AM5 and AM6 the cleavage pattern looked similar, the intensity of the band at G15 was higher than at G13 meaning higher accessibility of the enzyme to that nucleotide. For the antiterminators AM2, GlyQS and AM3, both G13
and G15 were cleaved in equal proportions. In C11U, there was cleavage at G13 but no cleavage at G15. The band intensities were normalized and relative intensities determined. Figure 2.8 shows the graphical presentation of the relative cleavage patterns. This data shows that there is flexibility in the G13 and G15 of the antiterminators AM1A, AM4, AM5 and AM6 and less flexibility for AM2, C11U, AM3 and GlyQS. T1 probing thus implies that the A2 helix structure differs between these models and is likely influenced by the adjacent loop structures.

2.2.3.2 RNase A probing

RNase A was also used to determine the secondary structure and the specific nucleotides that are not involved in base pairing or stacking. RNase A primarily cleaves the 3’ side of unpaired Cs (Brunel and Romby, 2000). Figure 2.9 and 2.10 shows the cleavage patterns of RNase A probing of the different antiterminators and Figure 2.11 shows the graphical presentation. In all the antiterminators, there was no cleavage in helix A1. Cleavage was observed of similar intensity at U6 in all the RNAs. There was also cleavage seen at G8 in AM1A, AM2, AM3, AM4 and GlyQS but with a lesser intensity. As with T1 probing, the major differences in the cleavage pattern were seen in the A2 helix, but in this case it was between the G13 and C14. The antiterminators AM1A, C11U, AM3 and AM4 had a similar pattern. In this case, C14 and G13 were cleaved in equal proportions. While for the antiterminators AM2, GlyQS, AM5 and AM6 C14 was cleaved more than G13. This data correlates well with the Mfold prediction thermostability and the Tm data. The high cleavage of C14 relative to G13 is correlated with the less negative $\Delta G$ and low Tm and low stability.
Figure 2.6 RNase T1 probing of antiterminators AM1A, C11U, AM2 (Wild type) and GlyQS (Glycine synthetase). The arrows indicate the different cleavage positions. Dark solid, strong cleavage (0.12-0.18), gray, medium cleavage (0.061-0.11) and open weak cleavage (0.01-0.06). The probing was performed in 10 mM Sodium phosphate buffer containing 1X structure buffer, 0.3 μg/μl of yeast RNA, 0.01U of RNase T1 and 0.25 μM 5’end labeled RNA.
Figure 2.7 RNase T1 probing of antiterminators AM3, AM4, AM5 and AM6. The arrows indicate the different cleavage positions. Dark solid, strong cleavage (0.12-0.18), gray, medium cleavage (0.061-0.11) and open weak cleavage (0.01-0.06). The probing was performed in 10 mM Sodium phosphate buffer containing 1X structure buffer, 0.3μg/μl of yeast RNA, 0.01U of RNase T1 and 0.25 μM 5’end labeled RNA.
Figure 2.8 Bar graphs of duplicate relative band intensities and the nucleotide position of T1 cleavage of different antiterminator structures for A1 helix, bulge and A2 helix. The relative band intensities of the average of two experiments were obtained by quantifying the intensity of each band in relation to the intensity of the entire lane.
Figure 2.9 RNase A probing of antiterminators AM1A, C11U, AM2 (Wild type) and GlyQS (Glycine synthatase). The arrows indicate the different cleavage positions. Dark solid strong cleavage (0.091-.013), gray, medium cleavage (0.041-0.09) and open weak cleavage (0.01-0.04). The probing was performed in 10 mM Sodium phosphate buffer containing 1X structure buffer, 0.3μg/μl of yeast RNA, 0.1ng of RNase A and 0.25 μM 5’end labeled RNA.
Figure 2.10 RNase A probing of antiterminators AM3, AM4, AM5 and AM6. The stars indicate the different cleavage positions. Dark strong cleavage (0.091-.013), open medium cleavage (0.041-0.09) and gray weak cleavage (0.01-0.04). The probing was performed in 10 mM Sodium phosphate buffer containing 1X structure buffer, 0.3μg/μl of yeast RNA, 0.1ng of RNase A and 0.25 μM 5’end labeled RNA.
Figure 2.11 Bar graphs showing relative band intensities of each nucleotide of RNase A cleavage of different antiterminator structures in the bulge and helix A2. The relative band intensities the averages of two experiments were obtained by quantifying the intensity of each band in relation to the intensity of the entire lane.
Figure 2.12 RNase V1 probing of antiterminators AM1A, C11U, AM2 (Wild type) and GlyQS (Glycine synthatase). The arrows indicate the different cleavage positions. Dark solid, strong cleavage (0.091-.013), gray, medium cleavage (0.041-0.09) and open weak cleavage (0.01-0.04. The probing was performed in 10 mM Sodium phosphate buffer containing 1X structure buffer, 0.3 μg/μl of yeast RNA, 0.0005U of RNase V1 and 0.25 μM 5’end labeled RNA.
Figure 2.13 RNase V1 probing of antiterminators AM1A, C11U, AM2 (Wild type) and GlyQS (Glycine synthatase). The arrows indicate the different cleavage positions. Dark solid, strong cleavage (0.091-.013), gray, medium cleavage (0.041-0.09) and open weak cleavage (0.01-0.04. The probing was performed in 10 mM Sodium phosphate buffer containing 1X structure buffer, 0.3 μg/μl of yeast RNA, 0.0005U of RNase V1 and 0.25 μM 5’end labeled RNA.
Figure 2.14 Bar graphs showing relative band intensities of each nucleotide of V1 cleavage of different antiterminator structures in A1 helix, bulge and A2 helix. The relative band intensities were obtained by quantifying the intensity of each band in relation to the intensity of the entire lane.
2.2.3.3 RNase V1 probing

RNase V1 probing was employed to monitor the base pairing and stacking interactions. V1 cleaves the paired and stacked regions (Brunel and Romby, 2000). Figure 2.12 and 2.13 shows the results from V1 cleavage of the different antiterminators. This result indicates helical formation in the A1 and A2 helix and stacking at the 3′end of the bulge. Apart from GlyQS that showed a less stacked A9 of the bulge. The graphical representation is shown in Figure 2.14

Having analyzed the effects of the loop on the structure of the antiterminator, it was necessary to investigate any effects on the function of the antiterminator. The affinity studies were carried out with tRNA and with microhelix UCCA. The role of monovalent and divalent metal ions in the binding studies was also investigated.

2.3 Affinity studies with tRNA

The affinity of different antiterminators to tRNA was tested along side the role of metal ions. As an RNA molecule folds into its native structure, the phosphate backbone is drawn together. A positively charged ion is required to neutralize the negatively charged metal ions. Monovalent metal ions are required to screen the negatively charged ion thus stabilizing the secondary structure (Basu et al., 1998; Shiman and Draper, 2000). Divalent metal ions are required for proper formation of the tertiary structure (Gonzalez and Tinoco, 2001; Tinoco and Bustamante, 1999).

To test the hypothesis that metal ions would differentially affect the tRNA affinity to different antiterminator models, the different antiterminator models were constructed with rhodamine fluorophore (Rhd) attached at the 5′ends of the antiterminators using an aminohexyl linker (Figure 2.15). The labeled antiterminators were designated 5′-Rhd-AM1A, 5′-Rhd-C11U, 5′-Rhd-AM2, 5′-Rhd-GlyQS, 5′-Rhd-AM3, 5′-Rhd-AM4 5′-
Rhd-AM5 and 5′-Rhd-AM6. The labeled, deprotected, desalted and PAGE purified RNAs were obtained from Dharmacon INC. These RNAs were dialyzed in 10 mM sodium phosphate buffer pH 6.5 and 0.01 mM EDTA. Due to the differences in the labeling efficiency (data not shown), it was necessary not to compare the absolute fluorescence intensity directly, but rather the relative change in the intensity upon binding the tRNA.

In all the fluorescence based binding assays involving 5′-Rhd antiterminators 100 nM each of the 5′-Rhd antiterminators were incubated in 50 mM sodium phosphate buffer (unless specified) pH 6.5, 50 mM NaCl and either 0 mM, 5 mM or 15 mM MgCl₂ and without or with the ligands in increasing concentrations of up to 2.6 µM for tRNA-UCCA and up to 312 µM for mhUCCA.

The fluorophore was excited at 540 nm with a cut off at 570 nm and emission spectrum collected between 570 and 640 nm. The fluorescence at 585 nm was analyzed which is the maximum fluorescence of Rhodamine. The normalized absolute relative fluorescence ($F_{rel}$) was determined from equation:

$$F_{rel} = \frac{|F-F_0|}{F_0}$$

Where by $F_{rel}$ is the relative fluorescence, $F$ the final fluorescence of 5′-Rhd antiterminators in complex with tRNA-UCCA at different concentrations and $F_0$ the initial fluorescence of 5′-Rhd antiterminators without the ligand (tRNA-UCCA).
**Figure 2.15** The structure of rhodamine fluorophore attached at position 5 of the ribose sugar using an amino hexyl linker. The fluorophore is attached at the 5’end of the antiterminator models.

To determine the binding affinities (dissociation constants $K_d$), the data was analyzed using the Graph Pad Prism version 4.0. The single site binding was compared to the two-site bind and plotted on the graph. In all cases, the single site binding was the preferred fit. The data in which the error was less than the $K_d$ with an $R^2$ (closeness of the points to the line of fit) of at least 0.8 was considered useful, otherwise was noted as no binding (n.b) or highlighted in bold.
2.3.1 The role of Mg\(^{2+}\) in tRNA-UCCA affinity for antiterminator AM1A and C11U

To determine the effects of Mg\(^{2+}\) on tRNA-UCCA affinity for antiterminator, the antiterminator AM1A and the least functional antiterminator C11U were investigated using the fluorescence based binding assays. A 5'-Rhd-AM1A or 5'-Rhd-C11U (100 nM each) were incubated in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl and either 0 mM, 5 mM or 15 mM MgCl\(_2\) and without or with tRNA in increasing concentrations up to 2.6 µM tRNA.

In both cases, the addition of tRNA-UCCA led to an increase in the directly observed fluorescence intensity at both 5 mM and 15 mM Mg\(^{2+}\), but there was no trend in the observed fluorescence intensity in the absence of Mg\(^{2+}\). Figure 2.16 shows the binding isotherms of F\(_{\text{rel}}\) plotted against tRNA-UCCA concentration in various concentration of Mg\(^{2+}\). Table 2.2 shows the binding affinities (K\(_d\)) of AM1A and C11U at different concentrations of Mg\(^{2+}\).

The data indicates that there was no binding at 0 mM Mg\(^{2+}\) for both AM1A and C11U. The best binding was seen at 5 mM Mg\(^{2+}\) for both antiterminators. At 15 mM Mg\(^{2+}\) there the binding affinity for AM1A was not so different from that at 5 mM. In C11U, there was no useful binding affinity at 15 mM Mg\(^{2+}\) due to a bad R\(^2\). From this data, it was also observed that Mg\(^{2+}\) was required for binding and that the binding affinity for AM1A is higher than for C11U. Also 5 mM Mg\(^{2+}\) was adequate for binding of tRNA-UCCA to the model AM1A.

2.3.2 Effects of Ca\(^{2+}\) in binding affinity

The effect of Ca\(^{2+}\) on tRNA affinity for AM1A was also determined. The experiment was prepared as above using the 5'-Rhd-AM1A alone. The binding buffer
was also 50 mM sodium phosphate pH 6.5, 50 mM NaCl, 0.01 mM EDTA and instead of magnesium chloride, calcium chloride was used. This experiment was not possible with the same concentration range of Ca\(^{2+}\) as that of Mg\(^{2+}\) due to precipitation of the buffers. Thus it was necessary to determine the best concentration to use in the experiment.

A calcium titration experiment was conducted on 5′-Rhd-AM1A and 5′-Rhd-C11U in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 0.01 mM EDTA and 0 to 200 µM of Ca\(^{2+}\) in the absence of tRNA. The best concentration of Ca\(^{2+}\) was estimated to be around 2.0 µM. An experiment was then conducted with the same buffer conditions but with a final concentration of 2.0 µM Ca\(^{2+}\) in the presence of various tRNA-UCCA concentrations. This experiment did not yield any useful data for either antiterminator model (data not shown). For AM1A the K\(_d\) was 0.19 but R\(^2\) was 0.4 indicating no binding. For C11U there was no convergence of the graph.

![Figure 2.16](image-url) The binding isotherms of tRNA-UCCA to 5′-Rhd-AM1A and 5′-Rhd-C11U at different concentrations of MgCl\(_2\). All binding assays were performed at 25 °C in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 0.01 mM EDTA and 0, 5, and 15 mM MgCl\(_2\).
Table 2.2 The effect of Mg$^{2+}$ on binding affinities of tRNA-UCCA to 5'-Rhd-AM1A and 5'-Rhd-C11U

<table>
<thead>
<tr>
<th>Model</th>
<th>$K_d$ µM ($R^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mM Mg$^{2+}$</td>
</tr>
<tr>
<td>AM1A</td>
<td>0.20±0.12(0.67)</td>
</tr>
<tr>
<td>C11U</td>
<td>n.b</td>
</tr>
</tbody>
</table>

The data in bold are considered as no binding due to a low $R^2$ value, n.b indicates data fit did not converge.

2.3.3 Effects of Cobalt hexamine III chloride

To evaluate the binding of metal ions through outer sphere mechanism, the effect of Cobalt Hexamine chloride on affinity of tRNA to 5'-Rhd-AM1A was tested. Like in the Mg$^{2+}$ experiment, the same buffer binding buffer conditions were used except that in the place of magnesium chloride, cobalt hexamine III chloride was used. The concentration of cobalt hexamine chloride varied between 0 and 2 mM. Figure 2.17 and Table 2.3 is the binding isotherms and binding affinities respectively. The data indicates that there was no binding at 0 mM. The binding was good up to 1.0 mM and the fit became poorer at 1.5 mM and 2.0 mM, most likely due to interference from precipitation of RNA in the buffer.

2.3.4 Effects of Sodium chloride

In order to determine whether monovalent metal ions are sufficient for stabilizing the antiterminator/tRNA complex, the 5'-Rhd AM1A was treated as the magnesium chloride study above. The buffer conditions were also as above but in this case Mg$^{2+}$ was eliminated completely and the NaCl concentration varied from 0 to 200
mM. The data was analyzed the same way. Figure 2.18 and Table 2.4 shows the binding isotherms and binding affinity respectively. This data shows that there was no binding even at the highest concentration of Na⁺.

**Figure 2.17** The binding isotherms of tRNA-UCCA to 5'-Rhd-AM1A. All binding assays were performed at 25 °C in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 0.01 mM EDTA and 0, 0.5 and 1.0 mM Co(NH₃)₆Cl₃ without MgCl₂.

**Table 2.3** The effect of cobalt hexamine chloride on binding affinities of tRNA-UCCA to 5'Rhd AM1A

<table>
<thead>
<tr>
<th>[Co(NH₃)₆Cl₃] mM</th>
<th>Kₐ µM (R²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.07 ± 0.08(0.28)</td>
</tr>
<tr>
<td>0.5</td>
<td>0.22 ± 0.07(0.86)</td>
</tr>
<tr>
<td>1.0</td>
<td>0.24± 0.06(0.92)</td>
</tr>
<tr>
<td>1.5</td>
<td>0.06±0.03(0.71)</td>
</tr>
<tr>
<td>2.0</td>
<td>0.57± 0.30(0.74)</td>
</tr>
</tbody>
</table>

The data shown in bold are considered as no binding due to bad R² or error larger than Kₐ.
Figure 2.18 A representative of binding isotherms of tRNA-UCCA to 5'-Rhd-AM1A in various concentration of NaCl. All binding assays were performed at 25 °C in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 0.01 mM EDTA and 0, 100, 150 and 200 mM NaCl.

Table 2.4 The effects of NaCl on binding affinity of tRNA-UCCA to 5'-Rhd-AM1A

<table>
<thead>
<tr>
<th>[NaCl] mM</th>
<th>$K_d$ µM ($R^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.03±0.04(0.39)</td>
</tr>
<tr>
<td>50</td>
<td>0.03±0.04(0.67)</td>
</tr>
<tr>
<td>100</td>
<td>0.008±0.03(0.19)</td>
</tr>
<tr>
<td>150</td>
<td>0.01 ± 0.03(0.27)</td>
</tr>
<tr>
<td>200</td>
<td>0.01 ± 0.03(0.22)</td>
</tr>
</tbody>
</table>

The data shown in bold are considered as no binding due to low $R^2$ or error larger than $K_d$. 
2.3.5 Effects of Mg\textsuperscript{2+} on other antiterminators

The effect of Mg\textsuperscript{2+} on tRNA-UCCA affinity for other antiterminators was investigated as in the previous Mg\textsuperscript{2+} study (section 2.3.1). The antiterminators tested were 5′-Rhd-AM2, 5′-Rhd-AM3, 5′-Rhd-AM4, 5′-Rhd-AM5, 5′-Rhd-AM6 and 5′-Rhd-GlyQS. These are the 5′-Rhd antiterminators with different loops and thermodynamic stabilities. All the reaction conditions were as previously described in section 2.3 and 2.3.1. Figure 2.19 and Table 2.5 shows the binding isotherms and binding affinities respectively. In all cases, there was no binding at 0 mM Mg\textsuperscript{2+}. Apart from the 5′-Rhd-AM1A and 5′-Rhd-AM4, the binding affinity for the different 5′-Rhd antiterminators was better at 15 mM than at 5 mM Mg\textsuperscript{2+}. Also the binding affinity correlated with Mfold thermodynamic stability prediction. With the more stable antiterminators having high affinity and the least stable having the lowest affinity. Interestingly though, the 5′-Rhd-AM2 had the best binding affinity at the highest Mg\textsuperscript{2+}. Even though it is the least stable from both MFold thermodynamic stability and T\textsubscript{M} studies.

2.3.6 Effect of pH on tRNA-UCCA affinity for antiterminator AM1A and C11U

As in the investigation of the effects of metal ions, the effect of pH was also studied using the fluorescence based binding assays. The fluorescently labeled RNAs were 5′-Rhd-AM1A and 5′-Rhd-C11U. The different pH and buffers were as previously described in section 2.2.2. The experiment was conducted as in the metal ions study except that instead of 50 mM sodium phosphate, 50 mM of the different buffers with various pH was used with 50 mM NaCl, 15 mM MgCl\textsubscript{2} and 0.01 mM EDTA. Table 2.6 and Figure 2.20 shows the binding affinities of tRNA-UCCA to 5′-Rhd-AM1A and 5′-Rhd-C11U in different pH and K\textsubscript{d} values of their complex with tRNA-UCCA versus pH respectively.
Figure 2.19 The binding isotherms of tRNA-UCCA to 5'-Rhd antiterminator models with different loop substitutions at 25 °C in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl and 0, 5 and 15 mM MgCl₂.
Table 2.5 The binding affinities of tRNA-UCCA to 5'-Rhd antiterminators with variations in the loop substitutions

<table>
<thead>
<tr>
<th>Model</th>
<th>K_d µM (R^2)</th>
<th>0 mM Mg^{2+}</th>
<th>5 mM Mg^{2+}</th>
<th>15 mM Mg^{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.08 ± 0.02 (0.84)</td>
<td>0.10 ± 0.02 (0.93)</td>
<td></td>
</tr>
<tr>
<td>AM1A</td>
<td>n.b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C11U</td>
<td>n.b</td>
<td>0.28 ± 0.07 (0.91)</td>
<td>0.18 ± 0.08 (0.73)</td>
<td></td>
</tr>
<tr>
<td>AM2</td>
<td>n.b</td>
<td>n.b</td>
<td>0.08 ± 0.02 (0.90)</td>
<td></td>
</tr>
<tr>
<td>AM3</td>
<td>n.b</td>
<td>0.13 ± 0.03 (0.87)</td>
<td>0.10 ± 0.03 (0.89)</td>
<td></td>
</tr>
<tr>
<td>AM4</td>
<td>n.b</td>
<td>0.10 ± 0.03 (0.85)</td>
<td></td>
<td>n.b</td>
</tr>
<tr>
<td>AM5</td>
<td>n.b</td>
<td>0.24 ± 0.06 (0.91)</td>
<td>0.18 ± 0.05 (0.91)</td>
<td></td>
</tr>
<tr>
<td>AM6</td>
<td>n.b</td>
<td>0.37 ± 0.10 (0.91)</td>
<td>0.16 ± 0.04 (0.92)</td>
<td></td>
</tr>
<tr>
<td>GlyQS</td>
<td>n.b</td>
<td>n.b</td>
<td>0.47 ± 0.20 (0.79)</td>
<td></td>
</tr>
</tbody>
</table>

The data noted as n.b. is where the R^2 was less than 0.8, the error greater than the K_d value or fit did not converge.
Table 2.6 The effect of pH on binding affinities tRNA-UCCA to AM1A and C11U determined by fluorescence based binding assays

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>$K_d$ µM ($R^2$)</th>
<th>AM1A</th>
<th>C11U</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES</td>
<td>5.0</td>
<td>0.07±0.02 (0.85)</td>
<td>n.b</td>
<td></td>
</tr>
<tr>
<td>MES</td>
<td>5.5</td>
<td>0.13±0.05(0.81)</td>
<td>0.13 ± 0.03(0.92)</td>
<td></td>
</tr>
<tr>
<td>MES</td>
<td>6.0</td>
<td>0.13±0.04 (0.89)</td>
<td>0.20 ± 0.07(0.85)</td>
<td></td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>6.5</td>
<td>0.10±0.02 (0.93)</td>
<td>0.18±0.08(0.73)</td>
<td></td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>6.75</td>
<td>0.32±0.25 (0.44)</td>
<td>Not measured</td>
<td></td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>7.0</td>
<td>0.26±0.04 (0.96)</td>
<td>0.05± 0.01(0.90)</td>
<td></td>
</tr>
<tr>
<td>Trizma</td>
<td>7.5</td>
<td>0.29±0.07 (0.89)</td>
<td>0.08 ± 0.02(0.85)</td>
<td></td>
</tr>
<tr>
<td>Trizma</td>
<td>7.75</td>
<td>0.33±0.09 (0.90)</td>
<td>Not measured</td>
<td></td>
</tr>
<tr>
<td>Trizma</td>
<td>8.0</td>
<td>0.52±0.12 (0.83)</td>
<td>0.37 ± 0.16(0.81)</td>
<td></td>
</tr>
</tbody>
</table>

The 5’Rhd AM1A (100 nM) and tRNA-UCCA (up to 2.6 µM) complex were incubated at 25 °C in 50 mM buffers Mes (pH, 5.0, 5.5, 6.0) Sodium phosphate (pH 6.5, 7.0) and Trizma (pH 7.5 8.0) containing 50 mM NaCl, 15 mM MgCl₂ and 0.01 mM EDTA. The data shown in bold means no binding due to poor $R^2$, n.b means the fit did not converge.
Figure 2.20 The binding affinities of tRNA-UCCA to AM1A and C11U versus pH determined by fluorescence based binding assays. The 5'Rdh AM1A (100 nM) and tRNA-UCCA (up to 2.6 µM) complex were incubated at 25 °C in 50 mM buffers Mes (pH, 5.0, 5.5, 6.0) Sodium phosphate (pH 6.5, 7.0) and Trizma (pH 7.5 8.0) containing 50 mM NaCl, 15 mM MgCl₂ and 0.01 mM EDTA.
The data indicates that the binding affinity of tRNA-UCCA to 5'-Rhd-AM1A decreased with higher pH. This data correlates well with the CD data above in section 2.2.2 whereby the stability in the phosphate backbone was perturbed with higher pH.

As in the CD study of C11U in section 2.2.2 the binding study did not show a clear trend with the changes in pH. The $K_d$ increased from pH 5.0 to 6.5 then decreased at pH 7.0 and 7.5 and jumped up again for pH 8.0 with a very big error. For both 5'-Rhd-AM1A and 5'-Rhd-C11U the $K_d$s were higher at pH 8.0. These results indicate that there could be protonation in AM1A but not in C11U.

### 2.4 2-Aminopurine studies of the antiterminators

Based on the findings from the 5'-Rhd labeled fluorescence binding studies another fluorophore was chosen in order to further investigate the conformation in the bulge region as a result of loop changes. In this study, 2-aminopurine was substituted in the 9th position of antiterminators AM1A, AM2 and GlyQS. The 2-Aminopurine (AP) is a base analog of Adenine and can perform the functions of A by base pairing with U (Zagorowska and Adamiak, 1996). The resultant models were designated as 9-AP-AM1A, 9-AP-AM2 and 9-AP-GlyQs respectively (Figure 2.21).

The experiment was done in a single well in a 600 µl quartz cuvette using the Fluoromax-3 Jobin Yvon Horiba instrument equipped with a temperature control. The instruments temperature was equilibrated at 20°C for all experiments. The fluorophore was excited at 310 nm and spectra collected between 330-600 nm at a rate of 1 second. Both the excitation and emission slit width were set to 5 nm. The binding studies of tRNA-UCCA to these models was investigated in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 5 mM MgCl$_2$ and 0.01 mM EDTA. TRNA-UCCA was titrated into these different fluorescently labeled antiterminators up to 15 µM tRNA.
The fluorophore was excited at 310 nm and scans collected between 330 nm and 600 nm. A background subtraction of buffer alone from scans containing labelled RNA was done. The titration of tRNA-UCCA into 100 nM of 9-AP-AM2 showed a decrease in the fluorescence up to 6.0 µM concentration, then the fluorescence increased again. A λ\text{max} shift of 4 nm to a longer wavelength was also observed in the spectra (Figure 2.22). For 9-AP-GlyQS the same trend was also seen. A reduction in spectra up to 10 µM and a shift of 4 nm was observed. But in this case the maximum emission wavelength was at 372 nm (Figure 2.23). In 9-AP-AM1A, the same trend was also observed as seen in 9-AP-AM2 (Figure 2.24).

The same experiment was also repeated with the same binding buffer conditions but with 15 mM Mg\textsuperscript{2+}. There was a decrease in fluorescence and a shift in all the antiterminators. The quenching of the fluorescence in 15 mM Mg\textsuperscript{2+} was much higher in 9-AP-AM1A (Figure 2.24) and 9-AP-AM2 than the one observed at 5 mM Mg\textsuperscript{2+} (Figure 2.22). For both these antiterminators, a shift of 4 nm was observed. The
fluorescence decreased at each addition of tRNA-UCCA to 9-AP-AM2. For 9-AP-AM1A, the fluorescence decreased up to 9.0 µM, then started to increase as observed in 5 mM. The spectra of tRNA-UCCA titrations into 9-AP-GlyQS in 15 mM Mg²⁺ did not show much difference from the 5 mM Mg²⁺ except that the shift in 15 mM Mg²⁺ was 6 nm (Figure 2.23).

Titrations of microhelix UCCA were also conducted on these antiterminators. The experiment was conducted as the tRNA titrations above. Except in this case, microhelix UCCA was added up to a final concentration of 140 µM. Different concentrations of Mg²⁺ was also investigated, the 5 mM and the 15 mM.

For the 5 mM Mg²⁺ the 9-AP-AM2, just like the 5 mM titrations of tRNA-UCCA, there was a decrease in fluorescence up to a concentration of 70 µM and then an enhancement with subsequent titrations. Also a shift to a longer wavelength of 4 nm was noticed (Figure 2.22). The titrations of mhUCCA to 9-AP-AM1A had previously been reported to show a 42% enhancement in fluorescence with mhUCCA titrations in 5 mM Mg²⁺. (Means, 2007b). The spectra of mhUCCA titrations into GlyQS in 5 mM MgCl₂ was similar to AM2 but with a shift of 10 nm to a longer wavelength in GlyQS (Figure 2.23).

In 15 mM Mg²⁺, titrations of mhUCCA to 9-AP-AM1A had been previously reported to induce a 12 nm shift in the emission peak. For 9-AP-AM2, unlike in the tRNA-UCCA titrations, there was a decrease up to a concentration of 70 µM, then an enhancement and a shift of 16 nm to a longer wavelength was also observed (Figure 2.22). For 9-AP-GlyQS, the same trend was observed and a shift of 14 nm (Figure 2.23).

In order to determine if the observed fluorescence was due to RNA/RNA interactions a control experiment was performed by tRNA-UCCA and mhUCCA titrations into the binding buffer (50 mM sodium phosphate pH 6.5, 50 mM NaCl, 0.01
mM EDTA and 5 or 15 mM MgCl\(_2\)) in the absence of the 9-AP antiterminators. The titrations of tRNA-UCCA into buffer showed some enhancement of the fluorescence at each titrations but this was not very significant (Figure 2.25). For the microhelix titrations, there was a decrease in fluorescence at a certain peak at about 345 nm and an enhancement at a peak of about 390 nM but all this was a very low intensity (Figure 2.25).

Another control experiments were performed by titrations of 10 mM sodium phosphate buffer pH 6.5, 0.01 mM EDTA into the 9-AP-AM2 and 9-AP-GlyQS. This is the buffer that all the RNA is dissolved in. The buffer was titrated in equal volumes as the ligands tRNA-UCCA and mhUCCA additions, which was a total of 20 µl. The titrations of 10 mM sodium phosphate buffer to each of these antiterminators did not show any significant change in the observed spectra (Figure 2.26).
Figure 2.22 The fluorescence spectra of tRNA-UCCA (A, B) or mh-UCCA (C, D) titrations into 9-AP-AM2 at 5 mM or 15 mM MgCl₂. The scans were measured at 20 °C in 50 mM sodium phosphate buffer pH 6.5, containing 100 nM of 9-AP-AM2, 50 mM NaCl, 0.01 mM EDTA and 5 mM MgCl₂ left spectra and 15 mM MgCl₂ right spectra. The tRNA-UCCA titrations were added up to 15 µM. While the mh-UCCA titrations were added up to 140 µM.
Figure 2.23 The fluorescence spectra of tRNA-UCCA (A, B) or mh-UCCA (C, D) titrations into 9-AP-GlyQS at 5 mM or 15 mM MgCl₂. The scans were measured at 20 °C in 50 mM sodium phosphate buffer pH 6.5, containing 100 nM of 9-AP-GlyQS, 50 mM NaCl, 0.01 mM EDTA and 5 mM MgCl₂ left spectra and 15 mM MgCl₂ right spectra. The tRNA titrations were added up to 15 µM. While the mh-UCCA titrations were added up to 140 µM.
Figure 2.24 The fluorescence spectra of tRNA-UCCA titrations into 9-AP-AM1A at 5 mM and 15 mM MgCl₂. The scans were measured at 20 °C in 50 mM sodium phosphate buffer pH 6.5, containing 100 nM of 9-AP-AM1A, spectra A, 5 mM and spectra B, 15 mM MgCl₂. The tRNA titrations were added up to 15 µM.
Figure 2.25 The fluorescence spectra of tRNA-UCCA (A, B) and mh-UCCA (C, D) titrations into buffer. The scans were measured at 20 °C in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 0.01 mM EDTA and 5 mM MgCl₂ spectra on the left and 15 mM MgCl₂ spectra on the right.
Figure 2.26 The fluorescence spectra of 10 mM sodium phosphate buffer titrations into 9-AP-AM2 (A, B) and 9-AP-GlyQS (C, D) at 5 mM or 15 mM MgCl2. The scans were measured at 20 °C in 50 mM sodium phosphate buffer pH 6.5, containing 100 nM of 9-AP-AM2 or 9-AP-GlyQS, 50 mM NaCl, 0.01 mM EDTA and 5 mM MgCl2 left spectra and 15 mM MgCl2 right spectra. A total of 20 µl of 10 mM sodium phosphate buffer pH 6.5, 0.01 mM EDTA was titrated in 10 additions.

2.5 Summary of quantitative analysis of the different antiterminators

From the different assays each antiterminator model was classified as either stable or less stable as compared to the average of all the models. Table 2.7 shows the different classifications. These classifications include the results from Mfold thermodynamic prediction in $\Delta G$ (kcal/mol), the CD by monitoring the $\lambda_{max}$ 268nm/209
nm ratio, RNase T1 G15: G13 ratio, RNase A C14:G13 ratio and their binding affinities to tRNA-UCCA. According to this classification, the antiterminator AM3 was classified as stable in all the assays. AM4 was also stable except in the binding assays and in the RNase T1 cleavage pattern. Antiterminators AM5 and AM6 were classified as less stable in all assays apart from the CD 268 nm: 209 nm ratio of model AM6 which was uncertain. However the classification of the wild type AM2 and AM1A and GlyQS hovered from stable to unstable or uncertain. But according to their binding affinity, the models AM1A and AM2 were classified as stable implying an induced fit binding.

Binding affinities versus Mfold predicted ∆Gs or versus melting temperature in 5 mM and 15 mM Mg^{2+} were also plotted (Figure 2.27). In 5 mM, there was no trend, but in 15 mM, the plot of binding affinity versus ∆G (kcal/mol) shows a trend between the binding affinity and the ∆G in the antiterminators AM1A, AM3, AM4, AM5 and AM6. The antiterminators AM2 and GlyQS were off the trend. Also in binding affinity versus melting temperatures, there was a trend in the antiterminators AM1A, AM3 and AM6 but again the antiterminators AM2 and GlyQS were off the trend. The lack of trend may be due to the absence of a stable tetraloop.
Table 2.7 Summary of quantitative analysis classification of the different antiterminator models compared to the average value in different assays

<table>
<thead>
<tr>
<th>Model</th>
<th>Assay</th>
<th>Quantitative analysis</th>
<th>Average quantitative value</th>
<th>Classification compared to average</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM1A</td>
<td>MFold (Kc atm/mol)</td>
<td>-13.5</td>
<td>-12.5</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>CD (268nm/209nm)</td>
<td>7.5/-7.4</td>
<td>8.4/-10.6</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>RNAse T1 (G15/G13) ratio</td>
<td>4.7</td>
<td>2.5</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>RNAse A (C14/G13) ratio</td>
<td>0.9</td>
<td>3.0</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Bind ing assays (µM)</td>
<td>0.1</td>
<td>0.12</td>
<td>X</td>
</tr>
<tr>
<td>AM2</td>
<td>MFold (Kc atm/mol)</td>
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<td>-12.5</td>
<td>X</td>
</tr>
<tr>
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<td>CD (268nm/209nm)</td>
<td>8.5/-8.3</td>
<td>8.4/-10.6</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>RNAse T1 (G15/G13) ratio</td>
<td>1.0</td>
<td>2.5</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>RNAse A (C14/G13) ratio</td>
<td>4.0</td>
<td>3.0</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Bind ing assays (µM)</td>
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<td>0.12</td>
<td>X</td>
</tr>
<tr>
<td>AM3</td>
<td>MFold (Kc atm/mol)</td>
<td>-13.9</td>
<td>-12.5</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>CD (268nm/209nm)</td>
<td>9.9/-13.2</td>
<td>8.4/-10.6</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>RNAse T1 (G15/G13) ratio</td>
<td>1.0</td>
<td>2.25</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>RNAse A (C14/G13) ratio</td>
<td>1.0</td>
<td>3.0</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Bind ing assays (µM)</td>
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<td>0.12</td>
<td>X</td>
</tr>
<tr>
<td>AM4</td>
<td>MFold (Kc atm/mol)</td>
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<td>-12.5</td>
<td>X</td>
</tr>
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<td></td>
<td>CD (268nm/209nm)</td>
<td>9.9/-13.2</td>
<td>8.4/-10.6</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>RNAse T1 (G15/G13) ratio</td>
<td>2.7</td>
<td>2.25</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>RNAse A (C14/G13) ratio</td>
<td>0.9</td>
<td>3.0</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Bind ing assays (µM)</td>
<td>0.1</td>
<td>0.12</td>
<td>X</td>
</tr>
<tr>
<td>AM5</td>
<td>MFold (Kc atm/mol)</td>
<td>-10.5</td>
<td>-12.5</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>CD (268nm/209nm)</td>
<td>7.7/-7.2</td>
<td>8.4/-10.6</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>RNAse T1 (G15/G13) ratio</td>
<td>2.5</td>
<td>2.25</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>RNAse A (C14/G13) ratio</td>
<td>4.4</td>
<td>3.0</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Bind ing assays (µM)</td>
<td>0.18</td>
<td>0.12</td>
<td>X</td>
</tr>
<tr>
<td>AM6</td>
<td>MFold (Kc atm/mol)</td>
<td>-12.5</td>
<td>-12.5</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>CD (268nm/209nm)</td>
<td>7.3/-14.6</td>
<td>8.4/-10.6</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>RNAse T1 (G15/G13) ratio</td>
<td>3.9</td>
<td>2.25</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>RNAse A (C14/G13) ratio</td>
<td>7.0</td>
<td>3.0</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Bind ing assays (µM)</td>
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<td>0.12</td>
<td>X</td>
</tr>
<tr>
<td>GlyQS</td>
<td>MFold (Kc atm/mol)</td>
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<td>-12.5</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>CD (268nm/209nm)</td>
<td>10.9/-11.5</td>
<td>8.4/-10.6</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>RNAse T1 (G15/G13) ratio</td>
<td>0.85</td>
<td>2.25</td>
<td>X</td>
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<tr>
<td></td>
<td>RNAse A (C14/G13) ratio</td>
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<td>X</td>
</tr>
<tr>
<td></td>
<td>Bind ing assays (µM)</td>
<td>0.47</td>
<td>0.12</td>
<td>X</td>
</tr>
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</table>
Figure 2.27 Binding affinities versus MFold predicted $\Delta G$ (kcal/mol) and versus melting temperature of different antiterminators in 5 mM (A, C) and 15 mM MgCl$_2$ (B, D). The graphs on the top show the binding affinity versus predicted $\Delta G$. While the graphs at the bottom shows the binding affinity versus experimental $T_M$.

2.6 Effects of ligands on fluorescence emission spectra of different antiterminators

The ligands tRNA-UCCA and neomycin were investigated for their effects on the fluorescence spectra of different antiterminator RNAs. The antiterminators studied were 5'-Rhd-AM1A, 5'-Rhd-C11U, 5'-Rhd-AM2 and 5'-Rhd-GlyQS. The study was conducted using the Flex station. A final concentration of 100 nM of each RNA sample was incubated in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 15 mM MgCl$_2$, ...
1 μM tRNA-UCCA and 200 μM neomycin. The fluorophore was excited at 540 nm with a cut off at 570 nm and emission spectrum collected between 570 and 640 nm. The readings were recorded after 10 min, 60 min and 120 min.

Figure 2.28 shows the plot of fluorescence versus the wavelength of different antiterminators with tRNA-UCCA or neomycin. As shown in the graphs, the tRNA-UCCA increased the fluorescence of all the antiterminators. While in the presence of neomycin, the fluorescence of the 5′-Rhd antiterminators decreased.

The fluorescence emission spectra of 5′-Fl-AM1A and 5′-Fl-C11U were also investigated in the absence or presence of tRNA-UCCA and neomycin. In this case the presence of both ligands decreased the fluorescence of both antiterminators (Figure 2.29). The decrease in fluorescence in the presence of tRNA-UCCA was much lower than in the presence of neomycin.
Figure 2.28 Fluorescence emission spectra of 100 nM 5'-Rhd antiterminators alone or in the presence of 200 µM neomycin or 1 µM tRNA-UCCA as indicated. The spectra were collected at 25 °C in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 5 mM MgCl₂ and 0.01 mM EDTA.
2.7 Initial ligand screening of the antiterminators

As a result of the differences in the A2 helix induced by loop differences that were observed in enzymatic probing, it was hypothesized that the different loops would affect the binding affinity of ligands. Different compounds were screened for their ability to bind different antiterminator model RNAs. These compounds included the oxazolidinones and the triazoles. The screenings was performed by fluorescence based binding assays. The antiterminator model RNAs tested were, 5’-Rhd-AM2, 5’-Rhd-GlyQS and 5’-Rhd-AM1A (S. Zhou and J. Hines unpublished results) all in a final concentration of 100 nM. The binding reaction consisted of 100 nM of each RNA in 50 mM sodium phosphate pH 6.5, 50 mM NaCl, 5 mM MgCl₂ and 0.01 mM EDTA and 10 µM of each compound. The normalized percentage of each compound was calculated.
(See section 5.11.4 of materials and methods) so as to determine the effect of the compound on the different antiterminators.

The compounds were compared for their effects on the change of fluorescence to each of the antiterminators. Figure 2.30 shows the normalized percentage change GlyQS versus AM2 of the oxazolidinones compounds. The dotted line shows the similarities in change for the compounds in the two RNAs. The compounds close to the line showed similar changes for both RNAs, and those that had a 15 % change in fluorescence were of interest. None of the oxazolidinones compounds showed similar changes to both the RNAs. More oxazolidinones compounds showed a greater change to GlyQS than to AM2. In the comparison between AM1A versus AM2 (Figure 2.31), only two compounds had a similar significant change for both the RNAs. More compounds showed a significant change for AM1A than AM2. None of the oxazolidinone compounds showed a similarity in fluorescence change for the comparison of AM1A versus GlyQS (Figure 3.32). But most compounds significantly changed the fluorescence of GlyQS.

For the triazole compounds, only one compound showed a significant change in fluorescence in similar way for GlyQS versus AM2 (Figure 2.33). Also like the oxazolidinones compounds, more compounds showed a significant change to GlyQS than AM2. The normalized percentage of AM1A versus normalized percentage AM2 of triazole compounds was also compared (Figure 2.34). Several compounds showed a similar change to both RNAs but only one compound showed a significant similar change in both RNAs. Most of the triazole compounds significantly changed the fluorescence of AM2 that AM1A. A comparison of AM1A versus GlyQS of the triazole compounds indicated that one compound significantly changed the fluorescence of
both RNAs in a similar manner (Figure 2.35). But most triazole compounds significantly changed the fluorescence of GlyQS.

From the initial screening study, it was observed that most oxazolidinone and triazole compounds more significantly change the fluorescence of GlyQS than AM2. The Oxazolidinone compounds preferentially change the fluorescence of AM1A than AM2 while the triazole compounds preferentially change the fluorescence of AM2 than AM1A. These differences in fluorescence change could be possibly due to the difference in RNA structure.
Figure 2.30 The % fluorescence difference of AM2 versus % fluorescence difference GlyQS of initial Screening of antiterminator models with oxazolidinones compounds (inset oxazolidinone core structure). The 100 nM 5'-Rh antiterminator AM2 and GlyQS models were screened with 10 µM each compound at 25 °C in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 5 mM MgCl₂ and 0.01 mM EDTA. (F-F₀/F₀)*100 is plotted where F= Fluorescence of labeled RNA in the presence of ligand (10 µM) and F₀ = fluorescence of labeled RNA in the absence of ligand.
Figure 2.31 The % fluorescence difference of AM2 versus % fluorescence difference AM1A of initial Screening of antiterminator models with oxazolidinone compounds (inset oxazolidinone core structure). The 100 nM 5'-Rhd antiterminator AM2 and AM1A models were screened with 10 µM each compound at 25 ºC in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 5 mM MgCl₂ and 0.01 mM EDTA.
Figure 2.32 The % fluorescence difference of GlyQS versus % fluorescence difference AM1A of initial Screening of antiterminator models with oxazolidinones compounds (inset oxazolidinone core structure). The 100 nM 5'-Rhd antiterminator GlyQS and AM1A models were screened with 10 µM each compound at 25 ºC in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 5 mM MgCl₂ and 0.01 mM EDTA. \((F-F_0/F_0)\times100\) is plotted where \(F\) = Fluorescence of labeled RNA in the presence of ligand (10 µM) and \(F_0\) = fluorescence of labeled RNA in the absence of ligand.
Figure 2.33 The % fluorescence difference of AM2 versus % fluorescence difference GlyQS of initial Screening of antiterminator models with triazole compounds (inset triazole core structure). The 100 nM 5’-Rhd antiterminator AM2 and GlyQS models were screened with 10 µM each compound at 25 ºC in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 5 mM MgCl₂ and 0.01 mM EDTA. \((F-F₀/F₀)*100\) is plotted where \(F\) = Fluorescence of labeled RNA in the presence of ligand (10 µM) and \(F₀\) = fluorescence of labeled RNA in the absence of ligand.
Figure 2.34 The % fluorescence difference of AM2 versus % fluorescence difference AM1A of initial Screening of antiterminator models with triazole compounds (inset triazole core structure). The 100 nM 5’-Rhd antiterminator AM2 and AM1A models were screened with 10 µM each compound at 25 °C in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, and 5 mM MgCl₂ and 0.01 mM EDTA. \((F-F_0/F_0)\times100\) is plotted where \(F\) = Fluorescence of labeled RNA in the presence of ligand (10 µM) and \(F_0\) = fluorescence of labeled RNA in the absence of ligand.
Figure 2.35 The % fluorescence difference of GlyQS versus % fluorescence difference AM1A of initial Screening of antiterminator models with triazole compounds (inset triazole core structure). The 100 nM 5'-Rhd antiterminator GlyQS and AM1A models were screened with 10 µM each compound at 25 ºC in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 5 mM MgCl₂ and 0.01 mM EDTA. (F-F₀/F₀)*100 is plotted where F= Fluorescence of labeled RNA in the presence of ligand (10 µM) and F₀ = fluorescence of labeled RNA in the absence of ligand.
2.8 Overall conclusions from all assays

In all assays, there was a correlation on the loop stability on the structure and functions of the antiterminators with a few exceptions. The Mfold prediction showed that the antiterminator AM2 was the least stable. This was consistent with the UV monitoring thermal denaturation where AM2 and GlyQS had the lowest $T_M$ even at high Mg$^{2+}$. On the other hand, the monovalent metal ions had the same effect on the $T_M$ for all the antiterminators with the exception of AM4, Mg$^{2+}$ had the highest effect on the antiterminator AM2.

In CD, the conformation of the different antiterminators also showed a correlation with their predicted stabilities with the exception of AM2 and the GlyQS. Analysis of the secondary structure using enzymatic proving showed some differences in the A2 helix but none or very little was observed in the bulge and the A1 helix. According to the single stranded specific RNase T1 cleavage, the cleavage pattern in the A2 helix showed some mixed observations. For example, the antiterminators AM1A, AM4, AM5 and AM6 showed a similar cleavage pattern where the G15: G13 ratio was found to be higher than the average of all the antiterminators. While in the models AM2, C11U, AM3 and GlyQS, the G13:G15 ratio was lower than the average. For RNase A probing, there was also differences in the cleavage pattern of the A2 helix and more in particular between the C14 and G13. Analysis of the C14: G13 ratio indicates that the models AM2, AM5, AM6 and GlyQS showed a similar pattern with a ratio higher than the average. The other models AM1A, C11U, AM4 and AM5 had a lower ratio of C14:G13. In this case RNase A cleavage pattern was much consistent with the stabilities of the different model. Whereby the higher ratio of C14:G13
cleavage correlated with less stability and the lower ration correlated with higher stability.

In the binding assays with the 5′-Rhd antiterminators, there was a much correlation between the stability of the model and its affinity with tRNA. The less stable antiterminators bind the tRNA with less affinity than the more stable antiterminators at both 5 mM Mg²⁺ and 15 mM Mg²⁺, but they bind better at the higher concentration of Mg²⁺. The model AM2 was exceptional, even though it is the least stable, it had the highest binding affinity with tRNA at the highest (15 mM) concentration of Mg²⁺.

Binding studies using the 9-AP antiterminators demonstrated a difference in binding with different concentrations of Mg²⁺. The 9-AP-AM1A and 9-AP-AM2 showed similar results at 15 mM Mg²⁺ for both tRNA-UCCA and mhUCCA binding. However, the binding of tRNA-UCCA to 9-AP-GlyQS was different from the other antiterminators even though the mhUCCA had shown similar results.

The binding of different compounds to 5′-Rhd-AM1A, 5′-Rhd-AM2 and 5′-Rhd-GlyQS indicated that more compounds were able to bind GlyQS and fewer compounds bind to and AM2. This may be as a result of difference in structure. All the assays demonstrated that the Stability and the conformation of AM1A and AM2 are different but their binding to tRNA-UCCA and mhUCCA was similar.
3 Results for structure-function studies of microhelix loop variants

3.1 Determination of model RNA stability

3.1.1 Mfold thermodynamic stability

Different microhelix model tRNA were constructed with changes in the loop region. Like the antiterminators, the different tetraloops were from the GNRA and the UNCG family. The resultant structures were folded and stabilities determined by using Mfold thermodynamic stability server version 3.2. Figure 3.1 shows the different constructs of the microhelix. The microhelix were classified into two groups. Those that were more stable than the microhelix UCCA and those that are less stable according to the Mfold thermodynamic prediction. Table 3.1 shows the \( \Delta G \) of the different microhelices. The microhelix designated as mh1 and mh2 had the GAAA and GCAA closing loops respectively and were predicted to be more stable than mhUCCA. While the microhelix mh3 and mh4 with closing loops UCCG and UGCG respectively were less stable than mhUCCA.

3.1.2 UV monitored thermal denaturation for determination of thermal stability

The stability of the different microhelix RNAs was determined. The experiment was conducted as previously described in the study of the antiterminators. The UV derivative plot in Figure 3.2 indicates that there was no complete denaturation in all the microhelices. This indicates that the melting temperatures of all the microhelices could be above 90 °C. The exact melting temperature of the microhelix could not be determined from this data because the RNAs did not reach a denatured phase where all the molecules are single stranded. The melting transition seemed to end at around
90 °C but it was not certain. Thus this assay was not very useful in determining the $T_m$ of the microhelices. This implies that the microhelices have a very strong tertiary structure under these buffer conditions.

![Figure 3.1](image)

**Figure 3.1** Microhelix model structures with different substitutions in the loop region: The thermal stability of each microhelix was predicted by Mfold thermodynamic stability.

**Table 3.1** The Mfold thermodynamic stabilities of different microhelix RNA models

<table>
<thead>
<tr>
<th>Microhelix</th>
<th>$\Delta G$ (kcal/mol) of desired structure</th>
<th>$\Delta G$ (kcal/mol) of close alternate structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mh1(GAAA)</td>
<td>-18.6</td>
<td>-13.3</td>
</tr>
<tr>
<td>Mh2(GCAA)</td>
<td>-18.6</td>
<td>-13.3</td>
</tr>
<tr>
<td>mhUCC A(UUCG)</td>
<td>-18.2</td>
<td>-12.9</td>
</tr>
<tr>
<td>Mh3(UCCG)</td>
<td>-14.4</td>
<td>-10.3</td>
</tr>
<tr>
<td>Mh4(UGC G)</td>
<td>-15.2</td>
<td>-10.3</td>
</tr>
</tbody>
</table>
Figure 3.2 The duplicate plots of UV derivative of microhelix RNA models with different loop substitutions. The UV melts were measured between 4 °C – 95 °C in 10 mM sodium phosphate buffer pH 6.5 and 0.01 mM EDTA. The different microhelices are: mh-UUCG, mh1-GAAA, mh2-GCAA, mh3-UCCG and mh4-UGCG.

3.2 Qualitative structural comparison

3.2.1 Circular dichroism (CD)

The conformation of the different microhelices was investigated using CD. Figure 3.3 shows the CD spectra obtained from scanning 10 µM of each mhUCCA, mh1, mh2, mh3 and mh4 in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 0.01 mM EDTA and either 5 mM or 15 mM MgCl₂. In spectra A where 5 mM MgCl₂ was used, there is no distinct difference at the 268 nm peak in all the microhelices, this
peak symbolizes the degree in base pairing and stacking. These results imply that the degree of base pairing and stacking is the same in all the microhelices. For the $\lambda_{\text{max}}$ 209 nm that symbolizes the stability in the phosphate backbone, the microhelices $\text{mh}_1$ and $\text{mh}_2$ with tetraloops GAAA and GCAA respectively had the most negative peak with a molar CD of -14.9 cm$^2$mmol$^{-1}$ and -14 cm$^2$mmol$^{-1}$ respectively. The molar CD for the $\text{mh}_{\text{UCCA}}$, $\text{mh}_3$ and $\text{mh}_4$ with closing tetraloops UUCG, UCCG and UGCG at $\lambda_{\text{max}}$ 209 nm was -8.0, -7.5 and -7.3 cm$^2$mmol$^{-1}$ respectively.

There was also not much difference at $\lambda_{\text{max}}$ 268 nm in 15 mM MgCl$_2$ for the microhelices, $\text{mh}_1$, $\text{mh}_3$, $\text{mh}_4$ and $\text{mh}_{\text{UCCA}}$. These are the microhelix with GAAA, UCCG, and UCCG tetraloops respectively. The molar CD for these tetraloops was about 11.5 cm$^2$mmol$^{-1}$ at $\lambda_{\text{max}}$ 268 nm. For the microhelix with the tetraloop GCAA ($\text{mh}_2$), the 268 nm peak was higher than the others with an intensity of 12.1 cm$^2$mmol$^{-1}$. In the case of the 209 nm peak, also $\text{mh}_1$ and $\text{mh}_2$ had a more negative peak of -13.5 and -14.5 cm$^2$mmol$^{-1}$ respectively. The rest of the microhelices $\text{mh}-3$, $\text{mh}-4$ and $\text{mh}-\text{UCCA}$ had a molar CD of about -8.0 cm$^2$mmol$^{-1}$. Again the loops that were predicted to be more stable are the ones that showed characteristics of stability in the phosphate backbone.
Figure 3.3 The CD spectra of average of duplicate scans of the different microhelix models with various loop substitutions; □ mh-UCCA (UUCG), ■ mh1-GAAA, ○ mh2-GCAA, ▲ mh3-UCCG and ▼ mh4-UGCG. The RNAs (10 μM) were scanned at 4 °C in 50 mM sodium phosphate buffer containing 50 mM NaCl, 0.01 mM EDTA and A, 5 mM MgCl₂ and B, 15 mM MgCl₂, in a final volume of 200 μl.
3.2.2 Enzymatic probing

Like in the enzymatic probing of the antiterminators, RNases T1, A and V1 were used to investigate the secondary structure of different microhelices. The microhelices in Figure 3.1 were labeled at the 5’ end using γ-P\(^{32}\) ATP and incubated in the different RNases. Figure 3.4 shows the cleavage pattern of RNase T1 probing. There was no cleavage in the mh-UCCA indicating a highly base paired secondary structure. For microhelices mh1, mh2 and mh4 there was weak cleavage at C7 of these microhelices. In the case of mh3 there was also cleavage at U8 and C9 in addition to C7. RNase T1 cleaves the 3’ end of unpaired Gs. Lack of any significant cleavage means that all the Gs are paired and stacked. There was no form of flexibility in the helical region as was noticed in some of the antiterminators.

Results from RNase A probing also did not show much cleavage in the microhelices (Figure 3.5). There was some cleavage in the nucleotides of the loop in mh2 and mh4. In the case of mh2 the nucleotides that were cleaved were G8 and C9. While in the case of mh4, the nucleotides cleaved were G9 and C10.

When RNase V1 was used for probing the microhelices, there was intense cleavage in the helical region that led to over cutting of the RNA. This prompted for the dilution of the enzyme 100X. Results from V1 probing using 1/100\(^{th}\) of the enzyme showed intense cleavage in mh1, mh2 and mh4 (Figure 3.6). This cleavage was mostly at G4, A5 and G6. There was also strong cleavage observed at C13 for mh1 and mh2. In mh4 strong
Figure 3.4 RNase T1 probing of microhelices UCCA, mh1, mh2, mh3 and AM4. The arrows indicate the different cleavage positions. Dark strong cleavage, gray medium cleavage and open weak cleavage. The probing was performed in 10 mM Sodium phosphate buffer containing 1X structure buffer, 0.3 μg/μl of yeast RNA, 0.01U of RNase T1 and 0.25 μM 5’end labeled RNA.
Figure 3.5 RNase A probing of mhUCCA, mh1, mh2, mh3 and AM4. The arrows indicate the different cleavage positions. Dark strong cleavage, gray medium cleavage and open weak cleavage. The probing was performed in 10 mM Sodium phosphate buffer containing 1X structure buffer, 0.3 μg/μl of yeast RNA, 0.01ng of RNase A and 0.25 μM 5’end labeled RNA.
Figure 3.6 RNase V1 probing of microhelices UCCA, mh1, mh2, mh3 and AM4. The arrows indicate the different cleavage positions. Dark strong cleavage, gray medium cleavage and open weak cleavage. The probing was performed in 10 mM Sodium phosphate buffer containing 1X structure buffer, 0.3 μg/μl of yeast RNA, 0.0005U of RNase V1 and 0.25 μM 5’end labeled RNA.
cleavage was also observed at both C12 and C13. The lack of cleavage using the single stranded specific RNase A and T1, and the extensive cleavage with RNase V1 in enzymatic probing of the different microhelices were very consistent with the T_m results where there was lack of complete denaturation.

3.3 **Affinity studies with antiterminator model AM1A**

3.3.1 **Effects of metal ions**

The effect of Mg^{2+} on binding of different microhelices to AM1A was investigated. The binding assays were performed using 100 nM 5′-Rhd-AM1A in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 0.01 mM EDTA and either 0, 5 or 15 mM MgCl_2. The microhelices investigated were mh1, mh2, mh3, mh4 and mh-UCCA. The binding isotherms are shown in Figure 3.7 and the binding affinities are shown in Table 3.2.

From this data, it was not necessary to compare the binding at 0 and 5 mM MgCl_2 because at those conditions, there was either no binding or a very high K_d. Thus the binding affinity at 15 mM MgCl_2 of all the microhelices was compared. Mh1 that has a closing loop of GAAA had the highest binding affinity followed by mh2 (GCAA) with an affinity mh-UCCA (UUCG), mh3 (UCCG) and finally mh4 (UGCG).

These data show that there was a correlation between loop stability and the binding affinity of different microhelices. The microhelices predicted to be most stable had the highest affinity than the less stable ones. The K_d of the different microhelices was plotted against the Mfold predicted ΔG (Figure 3.9). This graph shows that there is a trend between the stability and the affinity of the microhelices. With the more stable having the lowest K_d and the less stable with a high K_d.
Figure 3.7 The binding isotherms of microhelix model UCCA with different loop substitutions to 5'-Rhd AM1A at 25 °C in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl and 0, 5 and 15 mM MgCl₂.
Table 3.2 The effect of MgCl$_2$ on binding affinities of different microhelices to 5’-Rhd AM1A

<table>
<thead>
<tr>
<th>Model</th>
<th>K$_d$ µM ($R^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mM Mg$^{2+}$</td>
</tr>
<tr>
<td>mhUCCA</td>
<td>n.b</td>
</tr>
<tr>
<td>Mh1</td>
<td>n.b</td>
</tr>
<tr>
<td>Mh2</td>
<td>n.b</td>
</tr>
<tr>
<td>Mh3</td>
<td>n.b</td>
</tr>
<tr>
<td>Mh4</td>
<td>n.b</td>
</tr>
</tbody>
</table>

The data noted as n.b. is where the $R^2$ was less than 0.8 or the error greater than the K$_d$ value. All binding assays were performed at 25 ºC in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 0.01 mM EDTA and 0.5 and 15 mM MgCl$_2$.

3.3.2 Effects of Cobalt hexamine III chloride

The effects of cobalt hexamine chloride on the affinity of microhelix UCCA to 5’-Rhd-AM1A were investigated. Like in the Mg$^{2+}$ study, 100 nM of 5’-Rhd-AM1A was incubated in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 0.01 mM EDTA and either 0, 0.5, 1.0, 1.5 or 2.0 mM Cobalt hexamine III chloride. The microhelix UCCA concentration ranged from 0 to 312 µM. Figure 3.8 shows the binding isotherms of microhelix UCCA binding to 5’-Rhd-AM1A. The K$_d$s of each binding are summarized in Table 3.3.

No binding was observed in the absence of cobalt hexamine III chloride. The binding at 0.5, 1.5 and 2.0 mM did not make sense because it was terribly too high but with a good $R^2$ of 0.9, 0.97 and 0.96 respectively. For 1.0 mM concentration, the K$_d$
value was 676.8 µM, which was still too high than in comparison to when Mg$^{2+}$ is present.

### 3.3.3 Effect of pH on CD of microhelix UCCA and its complex with AM1A

Mh-UCCA and its complex with AM1A was scanned in various pH buffers of 5.0, 5.5, 6.0, 6.5 and 7.5. The various buffers used for each pH was based on the pKa of the buffer. Mes buffer was used for pH 5.0 and 5.5, sodium phosphate for pH 6.0 and 6.5 and Trizma for pH 7.5. A 50 mM concentration of each buffer also contained 50 mM NaCl, 15 mM MgCl$_2$ and 0.01 mM EDTA. In the complex, 10 µM of each RNA was heated at 90 ºC separately and cooled for 10 min then mixed to make a 1:1 ratio. The $\Delta\varepsilon$ was calculated as described in materials and methods. The individual experimental recorded CD of each RNA were added and the total $\Delta\varepsilon$ values determined. This summation was also plotted and compared to the experimental CD. All these spectra are shown in Figure 3.9. The results shows that both the microhelix UCCA and its complex with AM1A did not show any trend with the different pHs investigated an indication of no significant protonation effects on the overall structure. The CD of the microhelix alone and in complex with AM1A is different indicating a possibility of interaction. One fascinating feature observed was that the band intensity of the complex at $\lambda_{\text{max}}$ 268 nm decreased in the complex but the intensity at $\lambda_{\text{max}}$ 209 nm increased as compared to the summation of the spectrum and the mhUCCA alone implying a structural reorganization in the complex.
3.4 Summary of Quantitative analysis of the different microhelices

The summary was also done as in the antiterminators (Table 3.4). The enzymatic data was not included as it provided subtle information. In this analysis, mh1 and mh2 were classified as stable in most assays apart from the CD studies of mh1 in which it was uncertain. Both mh3 and mh4 were classified as less stable except the CD of mh4 that classified it as less stable. For microhelix UCCA, it was only classified stable by the Mfold thermodynamic prediction.

Also the binding affinity of the different microhelices in 15 mM Mg$^{2+}$ versus $\Delta G$ (kcal/mol) was plotted (Figure 3.10). Unlike in the antiterminators, the binding affinity versus $\Delta G$ for 5 mM Mg$^{2+}$ and binding affinity versus melting temperature was not included because no binding was obtained at 5 mM and also the melting temperatures of all the microhelices could not be determined correctly. This graph indicates a trend between $\Delta G$ and the binding affinities.

![Figure 3.8](image)

**Figure 3.8** The binding isotherms of microhelix model UCCA to 5’-Rhd AM1A at 25 °C in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl and 0, 0.5 and 1.0 mM Co(NH$_3$)$_6$Cl$_3$ without MgCl$_2$. 
Table 3.3 The effect of cobalt hexamine chloride on binding affinities of microhelix model UCCA to 5'-Rhd AM1A

<table>
<thead>
<tr>
<th>[Co(NH₃)₆]Cl₃ mM</th>
<th>Kₐ µM (R²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>n.b</td>
</tr>
<tr>
<td>0.5</td>
<td>34242± 431879(0.98)</td>
</tr>
<tr>
<td>1.0</td>
<td>676.8± 355.7(0.95)</td>
</tr>
<tr>
<td>1.5</td>
<td>1580±1280(0.97)</td>
</tr>
<tr>
<td>2.0</td>
<td>2407± 3448(0.96)</td>
</tr>
</tbody>
</table>

The data in bold is considered no binding due to error greater than the Kₐ value.
Figure 3.9 The CD spectra showing the effect of pH on CD of A, mhUCCA and AM1A alone and B, AM1A/mhUCCA complex. The scans were collected at 4 °C in 50 mM buffers; Mess for pH 5.0, 5.5 and 6, sodium phosphate buffer for pH 6.5 and Trizma for pH 7.5 with 50 mM NaCl, 15 mM MgCl₂, and 0.01 mM EDTA.
**Table 3.4** Summary of quantitative analysis classification of the different microhelix models compared to the average value in different assays

<table>
<thead>
<tr>
<th>Model</th>
<th>Assay</th>
<th>Quantitative analysis</th>
<th>Average quantitative value</th>
<th>Classification compared to average</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Stable</td>
</tr>
<tr>
<td>mhUCCA</td>
<td>MFold(Kcal/mol)</td>
<td>-18.2</td>
<td>-17</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>CD(268/209)</td>
<td>10.4/-8.0</td>
<td>10.6/-10.4</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Binding assays(Kd in μM)</td>
<td>78.85</td>
<td>64.3</td>
<td>X</td>
</tr>
<tr>
<td>mh1(GAAA)</td>
<td>MFold(Kcal/mol)</td>
<td>-18.6</td>
<td>-17</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>CD(268/209)</td>
<td>9.6/-13.5</td>
<td>10.6/-10.4</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Binding assays(Kd in μM)</td>
<td>30.85</td>
<td>64.3</td>
<td>X</td>
</tr>
<tr>
<td>mh2(GCAA)</td>
<td>MFold(Kcal/mol)</td>
<td>-18.6</td>
<td>-17</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>CD(268/209)</td>
<td>11.8/-14.2</td>
<td>10.6/-10.4</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Binding assays(Kd in μM)</td>
<td>60.26</td>
<td>64.3</td>
<td>X</td>
</tr>
<tr>
<td>mh3(UCCG)</td>
<td>MFold(Kcal/mol)</td>
<td>-14.4</td>
<td>-17</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>CD(268/209)</td>
<td>10.4/-7.8</td>
<td>10.6/-10.4</td>
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</tr>
<tr>
<td></td>
<td>Binding assays(Kd in μM)</td>
<td>123.5</td>
<td>64.3</td>
<td>X</td>
</tr>
<tr>
<td>mh4(UGCG)</td>
<td>MFold(Kcal/mol)</td>
<td>-15.2</td>
<td>-17</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>CD(268/209)</td>
<td>10.7/-8.3</td>
<td>10.6/-10.4</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Binding assays(Kd in μM)</td>
<td>153.78</td>
<td>64.3</td>
<td>X</td>
</tr>
</tbody>
</table>
Figure 3.10 The binding affinities versus the Mfold predicted thermodynamic stability of different microhelix models. The binding studies were conducted at 25 °C in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 15 mM MgCl₂ and 0.01 mM EDTA.
4 Discussion and Conclusions

4.1 The effect of metal ions and loop stability on T box antiterminator

Regulation of aminoacyl tRNA synthetases and other amino acid related genes relies on a unique RNA/RNA interaction called the T box antitermination mechanism (Henkin et al., 1992). About 805 genes have been identified in many Gram-positive bacteria (Vitreschak et al., 2008). Leader regions in these genes exhibit highly conserved primary and secondary structural elements (Grundy et al., 2000; Grundy et al., 2002a). Expression of the genes is achieved through the interaction of uncharged cognate tRNA with the first four nucleotides in the bulge of an antiterminator structural element in the conserved mRNA leader region (Grundy et al., 1994b). Formation of this structure is essential for complete transcription of the gene. The formation of an alternative terminator structure leads to transcription termination (Grundy and Henkin, 1994a).

The wide range of genes controlled by this mechanism makes it an intriguing mechanism for potential drug targeting. Phylogenetic and mutational studies have shown that the loop region in the antiterminator lacks conservation both in sequence and size (Grundy et al., 2002a). This region in the antiterminator model AM1A was replaced with the most stable and well-characterized UUCG tetraloop (Gerdeman et al., 2002). Binding studies involving this model showed that the A2 helix of the antiterminator becomes more open upon binding, hence less structural A form geometry (Anupam, 2007).

This study investigated the significance of this deformation. In this study, computational, spectroscopic and molecular techniques indicate that the loop stability had an effect on the structure and functions of the antiterminator. The investigation of
the role of metal ions on the tRNA affinity for the antiterminator indicated that divalent metal ion are required for proper folding and binding of tRNA to the antiterminator and that Mg$^{2+}$ was the most preferred.

Loops in RNA are known to play an important part in the folding of an RNA molecule. The tetraloops are known to form very stable structures (Antao et al., 1991). The UNCG and the GNRA have been well characterized as the most prevalent and stable tetraloops forming about 70% of the known tetraloops (Uhlenbeck, 1990; Woese et al., 1990). In order to determine the effects of the loop stability, Antiterminators with different loop regions were generated. The Mfold prediction of the resultant antiterminators indicated the significance of the loop stability on the general stability of an RNA molecule. The low melting temperature of the antiterminators AM2 and GlyQS as compared to the other antiterminators indicates that indeed the UNCG and the GNRA are the most stable and the tetraloop contributes a lot of stability to the antiterminator structure. The GlyQS and the AM2 do not have this kind of tetraloops. In GlyQS, though it contains the tetraloop sequence GAAC in which only C4 makes it different from a typical GNRA tetraloop. This indicates the degree of conservation in the tetraloop that enables its stability. Studies show that in the GNRA tetraloops, G1 forms base pairs with A4 and there are also several 2′ OH-base hydrogen bonds (Sorin et al., 2002; Williams and Hall, 2000). In GAAC tetraloop of GlyQS, a single nucleotide difference likely prevents it from forming a typical GNRA tetraloop and attaining the general loop stability.

In the CD studies (Figure 2.4), the GlyQS had the highest intensity at the $\lambda_{\text{max}}$ 268 nm. This peak usually symbolizes the degree of base pairing and stacking. A structure with a high intensity peak would be thought to be the most stable. But in this case the high intense peak was likely due to more base pairing in the A2 helix as
compared to the other antiterminators. GlyQS has 6 base pairs compared to 5 in AM2 and 4 in the other antiterminators. Also the intensity at $\lambda_{\text{max}}$ 268 nm for AM2 was unexpected. Being the least stable antiterminator as assayed by Mfold prediction and the melting temperature studies, the results from the CD indicated a more intense peak at 268 nm compared to the rest of antiterminators. This also was most likely due to the extra base pairing. Higher Mg$^{2+}$ concentration did not change the CD of the new antiterminator model significantly indicating the lack of Mg$^{2+}$ dependent structural reorganization as previously reported for AM1A and C11U (Jack et al., 2008).

Enzymatic probing indicated that the loop region mostly affects the A2 helix but not the nature of the bulge or the A1 helix. The A2 helix becomes flexible in the presence of a very stable loop region. This is evident in T1 cleavage of G13 and G15 of the antiterminators AM1A, AM4, AM5 and AM6. Even though this followed the trend, AM3 was exceptional.

The link between structure and binding affinity of the different antiterminators was tested using fluorescence based binding assays. It was hypothesized that the most stable structures would have a high binding affinity. In one assay, the 5'-Rhd antiterminators were used. The role of metal ions was also investigated. It was evident in this study that metal ions plays an important role in the folding and binding of the antiterminator RNA to tRNA. Monovalent metal ions are not sufficient for the binding of the tRNA-UCCA to the antiterminator AM1A. When binding studies were conducted using the 5'-Rhd-antiterminators in the presence of Na$^+$, no binding was observed even at the highest concentration of Na$^+$. This led to the conclusion that the binding of tRNA-UCCA to the antiterminator required the presence of divalent metal ions.
To test for the role of other metal ions, CaCl₂ was employed. Ca²⁺ has been demonstrated to perform the function of Mg²⁺ by enhancing the binding of tRNA to RNase P RNA (Smith et al., 1992). When this metal ion was substituted in the place of Mg²⁺ in the binding studies of tRNA-UCCA with 5′-Rhd-AM1A, no binding was observed. The same concentration of Ca²⁺ as used for Mg²⁺ could not be used due to precipitation of the Ca²⁺ in the presence of phosphate ions in the 50 mM sodium phosphate buffer that was used for binding. When the lowest concentration was used of Ca²⁺ was used, no binding was also observed. As a result this metal ion was dropped as a probe for the role of Mg²⁺.

To probe for diffuse binding over specific binding, cobalt hexamine III chloride was used. The results from this study indicated binding in the presence of cobalt hexamine III chloride in the absence of Mg²⁺. The binding was only good up to 1 mM concentration then became poorer at higher concentrations. This was consistent with the findings from (Jack et al., 2008) in the identification of Mg²⁺ binding sites. The binding of tRNA-UCCA to AM1A required lower concentrations of cobalt hexamine III chloride than for Mg²⁺ probably due to the higher charge on the cobalt hexamine III chloride.

Mg²⁺ was shown to play an important role in the binding studies. No binding was observed in the absence of Mg²⁺ in all the antiterminators (Table 2.5). The 5 mM Mg²⁺ was sufficient for tRNA-UCCA binding to the antiterminators AM1A, AM3 and AM4. Increasing the concentration of Mg²⁺ to 15 mM did not significantly change the binding affinity of these antiterminators. This is consistent with the studies involving the GlyQS leader region that demonstrated that Mg²⁺ is required for both folding of the GlyQS leader region and interaction with tRNA₅Gly. A threshold of Mg²⁺ was required for efficient tRNA directed antitermination (Grundy et al., 2002). Absence of binding was observed at 0 mM Mg²⁺ but sufficient binding was observed at 5 mM and maximum at 15 mM
concentration of Mg\textsuperscript{2+}. Higher concentration of Mg\textsuperscript{2+} above 15 mM did not change the binding affinity significantly (Yousef et al., 2005).

Also noticed in the role Mg\textsuperscript{2+} in the binding affinity of tRNA-UCCA to the different antiterminators was that the loop region played an important role. The antiterminator AM2, AM5 and AM6 which were considered to be less stable showed their best binding at the highest concentration of Mg\textsuperscript{2+}. This indicates that these antiterminators required Mg\textsuperscript{2+} for stabilization of the complex. The most effect of high Mg\textsuperscript{2+} was seen in the antiterminator AM2. One reason for this could be due to the flexibility of this loop such that the number of conformations that the tRNA can sample is high. These results are consistent with the melting temperature studies, whereby an increase in Mg\textsuperscript{2+} from 5 mM to 15 mM had the highest effect on the melting temperature of AM2. The melting temperature of AM2 was raised by 6 °C compared to the other antiterminators which were raised by between 1 °C to 4 °C. These findings are also consistent with the foot printing of the group I introns from Azoarcus pre-tRNA\textsuperscript{Ile} that showed that tertiary interactions between the helical domains were stable in a variety of cations but Mg\textsuperscript{2+} induced a conformational change in the introns core that correlated with self-splicing activity (Rangan and Woodson, 2003). The high dependence of AM2 on Mg\textsuperscript{2+} compared to the other antiterminator models could also imply a possibility of a Mg\textsuperscript{2+} binding site inducing the loop of AM2 or a that there is a Mg\textsuperscript{2+} binding site in the loop.

Studies in which the 2-aminopurine fluorophore was used to investigate the conformational changes in the bulge region indicate that there was no net enhancement of the fluorescence when microhelix-UCCA was titrated into 9-AP-AM2 and 9-AP-GlyQS in both 5 mM and 15 mM Mg\textsuperscript{2+}. Instead there was a shift indicating stacking around the fluorophore.
The tRNA titrations into the 9-AP antiterminators also showed a decrease in the fluorescence up to a certain concentration then an enhancement. The shift was most pronounced at 15 mM Mg\(^{2+}\) indicating the role of Mg\(^{2+}\) in the binding. The NMR study of the antiterminator model AM1A indicates that A9 is stack with the other nucleotides in the 3' end of the bulge (Gerdeman et al., 2003). The present results are consistent with the induced fit that was earlier proposed (Gerdeman et al., 2003) and the two step binding process (Means, 2007b) in which the first three nucleotides of the bulge binds first to the tRNA, then a conformational changes occurs that allows A9 to bind forming a base pair with the discriminator base. This conformational change is evident by the enhancement of the fluorescence when a certain concentration is achieved. The binding is also facilitated by high concentration of Mg\(^{2+}\). The same trend was observed in the investigations where 2-aminopurine was used to monitor the structural changes of antiterminator AM1A binding to model tRNA (Means, 2007b).

The pH studies indicated a likelihood of base protonation in the model AM1A but not C11U. The only difference between AM1A and C11U is the pyrimidines switch at position 11 from a C to a U. These results indicate that C11 is most likely to be protonated. Similar mutations were reported to have reduced the self-cleavage activity of the HDV ribozyme at C41 sequence that is pH sensitive (Wadkins et al., 2001). C41, which participates in tertiary interactions, is protonated at its N-3 position at neutral pH (Ferre-D'Amare and Doudna, 2000). Mutation of C41U that would retain features in the absence of protonation of wild type protonated tertiary interaction resulted in a variant with low activity at low pH. This decrease in activity was shown to be as a result of lack of protonable base. Similarly the protonated base in AM1A was likely to stabilize the RNA model and therefore increase its binding affinity. The stabilization of model AM1A by a protonated base slightly affected the stability in the phosphate backbone.
Phosphate groups have been shown to stabilize the HDVR by elevating the pKas (Tang et al., 2007). In the HDVR the protonation of C75 in the active site is brought together with adjacent phosphate groups that eventually leads to its stabilization.

4.2 Effect of metal ions and loop stability on mhUCCA

The microhelix-UCCA was constructed in order to investigate other features of tRNA that are essential for antitermination (Gerdeman et al., 2002). This construct consisting of the acceptor stem of the tRNA was also closed with the UUCG tetraloop. Previous studies had shown evidence of flexibility in the acceptor end of the tRNA upon binding to the antiterminator (Agyeman, 2007). The significance of this deformation was investigated.

The Mfold prediction shows a correlation between the loop stability and the $\Delta G$ of the structure indicating the contribution of the loop on the stability. Even though the melting temperature indicated not much difference in the stability of these difference microhelices. The CD data indicated different conformations. The contribution of this stability was mostly observed in the phosphate backbone and not in the stacking or tertiary structure formation. This was evident from the CD data whereby mh1 and mh2 for microhelices closed with GAAA and GCAA tetraloop respectively had a very negative peak at $\lambda_{\text{max}}$ 209 nm (Figure 3.3) indicating stability in the phosphate backbone. The 268 nm peaks did not show much significant differences among the different microhelices.

The enzymatic and melting temperature analysis indicates that the different microhelices have a very rigid tertiary structure. The lack of cleavage in the helical region with single strand specific RNase T1 and RNase A clearly indicates the inaccessibility of the enzyme to these regions. The increased cleavage with the double
stranded specific RNase V1 clearly indicates a stable helical structure. In the melting temperature analysis, there was no complete denaturation of the microhelices at above 90 ºC (Figure 3.2). This also indicates an evidence of a strong tertiary structure.

The effect of the loop on binding affinity of the different microhelices was also tested. In this investigation, the 5’-Rhd-AM1A was incubated in different microhelices. Also the role of Mg$^{2+}$ in the binding affinity was investigated. The results indicate that the loop stability affects the affinity of the microhelix to the antiterminator. As it was hypothesized the microhelix with the stable tetraloop had the highest binding affinity while the ones with the least stable tetraloop had the lowest binding affinity. Like in the antiterminators, Mg$^{2+}$ also played an important role in the binding affinity. This suggests that the microhelix undergoes some conformational changes upon binding. This conformation is facilitated by the presence of Mg$^{2+}$ and the loop stability. The conformational changes that occur when mhUCCA binds AM1A were also demonstrated by the effect of pH on the CD of the complex (Figure 3.9). The summation of the complex had a $\Delta \epsilon$ of higher intensity at $\lambda_{\text{max}}$ 268 than the experimental. In contrast with the effect of pH on AM1A, the mhUCCA alone and in complex with AM1A did not show any possibility of protonation.

4.3 Overall conclusions

This study demonstrated that the loop had an effect on the structure and the functions of both the antiterminator and the microhelix. The substitution of the AAUCA loop with the most stable UUCG tetraloop had a subtle effect on the structure of the antiterminator. But this did not alter the nature of the bulge or its binding affinity to tRNA. The binding of the antiterminator AM1A and AM2 to tRNA-UCCA is facilitated by induced fit or tertiary structure capture. This induced fit is enhanced by high Mg$^{2+}$ concentration.
This induced fit was earlier hypothesized in the NMR study of the antiterminator AM1A (Gerdeman et al., 2003) and further evidence was provided (Anupam, 2007; Means, 2007b).

The conformational flexibility of the AAUCA loop in the antiterminator AM2 compared to the more stable UUCG may allow increased sampling of those conformations with high binding affinity at high concentration of Mg$^{2+}$. This idea of conformational flexibility was also reported by (Persson et al., 2002). When they compared the UUUU and the GNRA tetraloops for their catalytic function of the hammerhead ribozyme. Though the UUUU tetraloop was thermodynamically less stable than the GCAA tetraloop, the UUUU was found to be catalytically more active due to increased sampling resulting from its conformational flexibility.

It was also demonstrated in this research that Mg$^{2+}$ is essential for folding and functions in the T box antitermination mechanism and monovalent metal ions alone are not sufficient. This was similar to what was demonstrated in the stabilization of the tertiary structure of ribozymes in the presence of high concentrations of monovalent metal ions (Pyle, 1993).

Due to the uniqueness of the novel T box antitermination mechanism, it is a possibility of becoming a future drug target. Given this feature of the wild type antiterminator AM2 and its ability to bind tRNA better than other antiterminators, it would be the best antiterminator for initial ligand screening. This study has identified structural differences in the loop region that result in the ultimate function of the antiterminator thus contributed to the overall understanding of the T box antitermination mechanism.
4.2 Future directions

Since the wild type antiterminator model AM2 has proved to be functional in vitro, it will be necessary to characterize ligand binding to this model and compare it with the ligand binding to AM1A. It will also be important to study the NMR structure of AM2 in order to obtain its clear structure as well as investigate the possibility of a Mg\(^{2+}\) binding site.

It will be necessary to use a better assay for the initial screening of the compounds. Different compounds responded differently on the 5′-Rhd-antiterminators. In some, there was enhancement of the fluorescence and in others reduction. Hence it was not easy to make a judgment on the most selective compound. Finally, it will be necessary to characterize the compounds that were selected in the initial screening.
5 Materials and methods

5.1 General procedures

All procedures involving handling of the RNA were done in sterile conditions. Gloves were worn all the time when handling RNA in order to protect the RNA from RNases. The glassware used was washed with warm tap water and mild soap and rinsed several times with ultra purified water obtained from Barnstead NaNopure diamond™ (UV/UF) purifying system. The glassware was left to dry up on the rack then covered with aluminum foil and oven dried at 80 °C overnight. The dry glassware was then kept in the cabinets until use.

The buffers used were obtained from Sigma Aldrich, Fisher or VWR and were either molecular biology grade or high quality grade. All buffers were prepared using ultra purified water and autoclaved for 25 min using a Consolidated MK II Controller obtained from Consolidated Still and Sterilizers unless specified in appendix.

All UV readings were taken using the Beckman 530 UV-VIS. The absorbance of DNA templates, RNA and primers at wavelength 260 nm, 280 nm, and 320 nm (A_{260}, A_{280} and A_{320} respectively) were determined using the program NucleicA_{260}. The corrected value of the absorbance (A_{cor}) was calculated by subtracting the A_{320} readings from each A_{260} and A_{280}, since nucleic acids do not absorb in this region. Any absorbance would mean dirty cuvettes or the presence of dust particle (Ganske, 2008). The ODs of DNA template, primers and RNA samples was calculated according to the following equation:

$$\text{OD} = A_{260\text{cor}} \times \text{dilution factor} \times V_{\text{ml}}.$$ 

Where by A_{260\text{cor}} is the corrected A_{260}, dilution factor is the total volume in ml in the UV cuvette, divided by Volume of sample added to the cuvette and V_{ml} is the initial volume.
of sample in ml. The concentration was calculated from ODs using the conversion factor of the appropriate sample (Table 5.1) provided by IDT or calculations made from Oligo Tech program version 1.0. In case of the fluorescently labeled RNA samples, the concentration was calculated using the extinction coefficients provided by Dharmacon (Table 5.2).

Table 5.1 The conversion factors of different DNA templates and RNA

<table>
<thead>
<tr>
<th>DNA templates</th>
<th>Conversion factor from IDT(nM/OD)</th>
<th>RNA</th>
<th>Conversion factor from oligo Tech calculations (nM/OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM1A</td>
<td>2.2</td>
<td>AM1A</td>
<td>3.75</td>
</tr>
<tr>
<td>C11U</td>
<td>2.2</td>
<td>C11U</td>
<td>3.77</td>
</tr>
<tr>
<td>AM2</td>
<td>2.1</td>
<td>AM2</td>
<td>3.18</td>
</tr>
<tr>
<td>GlyQS</td>
<td>2.1</td>
<td>GlyQS</td>
<td>3.17</td>
</tr>
<tr>
<td>AM3</td>
<td>2.3</td>
<td>AM3</td>
<td>3.60</td>
</tr>
<tr>
<td>AM4</td>
<td>2.3</td>
<td>AM4</td>
<td>3.62</td>
</tr>
<tr>
<td>AM5</td>
<td>2.0</td>
<td>AM5</td>
<td>3.79</td>
</tr>
<tr>
<td>AM6</td>
<td>2.3</td>
<td>AM6</td>
<td>3.65</td>
</tr>
<tr>
<td>Mh-UCCA</td>
<td>2.6</td>
<td>Mh-UCCA</td>
<td>5.05</td>
</tr>
<tr>
<td>Mh-1</td>
<td>2.7</td>
<td>Mh-1</td>
<td>4.78</td>
</tr>
<tr>
<td>Mh-2</td>
<td>2.7</td>
<td>Mh-2</td>
<td>4.92</td>
</tr>
<tr>
<td>Mh-3</td>
<td>2.6</td>
<td>Mh-3</td>
<td>5.08</td>
</tr>
<tr>
<td>Mh-4</td>
<td>2.6</td>
<td>Mh-4</td>
<td>5.12</td>
</tr>
<tr>
<td>Transcription Primer</td>
<td>5.5</td>
<td>tRNA-UCCA</td>
<td>1.25</td>
</tr>
<tr>
<td>3‘tRNAPrimer</td>
<td>3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5‘tRNA primer</td>
<td>6.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The conversion factors of the DNA templates and primers are recorded as provided by IDT, while the conversion factors of the RNAs was calculated from Oligo Tech program as indicated.
Table 5.2 The extinction coefficients of different fluorescently labeled RNAs as provided by Dharmacon

<table>
<thead>
<tr>
<th>Fluorescently labeled RNAs</th>
<th>Extinction coefficient (L/mole.cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′-Rhd-AM1A</td>
<td>299,000</td>
</tr>
<tr>
<td>5′-Rhd-C11U</td>
<td>302,300</td>
</tr>
<tr>
<td>5′-Rhd-AM2</td>
<td>346,780</td>
</tr>
<tr>
<td>5′-Rhd-AM3</td>
<td>310,200</td>
</tr>
<tr>
<td>5′-Rhd-AM4</td>
<td>308,300</td>
</tr>
<tr>
<td>5′-Rhd-AM5</td>
<td>296,300</td>
</tr>
<tr>
<td>5′-Rhd-AM6</td>
<td>306,400</td>
</tr>
<tr>
<td>5′-Rhd-GlyQS</td>
<td>347,580</td>
</tr>
<tr>
<td>9-AP-AM1A</td>
<td>257,400</td>
</tr>
<tr>
<td>9-AP-AM2</td>
<td>305,500</td>
</tr>
<tr>
<td>9-AP-GlyQS</td>
<td>306,300</td>
</tr>
</tbody>
</table>

5.2 Preparations of T7 Polymerase

The T7 polymerase that was used in the transcriptions of RNA in the lab was prepared as previously described (Agyeman, 2007; Jack, 2007).

5.3 RNA design

The antiterminator RNAs, tRNA-UCCA and microhelix-UCCA models were designed based on the Bacillus subtilis tyrS gene (Gerdeman et al., 2002). Their analogs were substituted in the loop region with different tetraloops belonging to the GNRA and UNCG family (Tuerk et al., 1988a; Woese et al., 1990). The GlyQS antiterminator was based on the B. subtilis Glycine synthetase gene. The secondary structures were predicted as described in section 5.7.

Fluorescently labeled antiterminator RNAs included 5′-Fluorescein end labeled, 5′-Rhodamine end labeled, 3′-Fluorescein-U18-Rhodamine labeled (FRET-labeled)
and 2-aminopurine internally labeled RNAs. The 2-aminopurine internally labeled RNAs contained 2-aminopurine substitutions for A at position 9 of the antiterminators.

5.4 Preparation of antiterminator RNA and microhelix

5.4.1 Preparation of DNA template and primers

RNA was prepared by in vitro transcription from the DNA template and primers obtained from Integrated DNA technologies (IDT) using T7 polymerase (Milligan et al., 1987; Milligan and Uhlenbeck, 1989). The sequence of the primer used for transcription of all antiterminator RNAs and microhelices was 5′-TAATACGACTCTATAG-3′. The sequence for the DNA template varied depending on the RNA to be transcribed. The lyophilized DNA template or primer was first dissolved in about 100 µl of distilled water and mixed thoroughly by vortexing. Equal amounts of gel loading buffer without dye (Appendix A) was then added. The sample was denatured by heating at 90 °C for 1.5 min before loading on a preheated 20% denaturing polyacrylamide (19:1 acrylamide: bis acrylamide) gel. The gel was run at 30 Watts and 1100 Volts. Due to differences in number of nucleotides, the DNA template was allowed to separate for 14 hours while the primers were allowed to separate for 8 hours, after which the gel was removed and wrapped in Fisher brand all-purpose polyvinyl chloride wrap. The bands were identified and excised by placing a fluorophore impregnated TLC plate under the gel and visualized by UV shadowing with a long Life™ filter with short wave ultra violet 254 nm obtained from Spectroline.

The sample was eluted from the bands using the elutrap electrophoresis system obtained from Schleicher & Schuell Bioscience. The electrophoresis traps were assembled with the appropriate membranes (BT1 and BT2) to form a collection chamber. The gel bands were cut into small pieces and placed into one chamber.
While 1X autoclaved TBE (50 mM Tris-Borate pH 8, 1 mM EDTA) buffer was added to another trap with two spacers separating the BT1 and BT2 membranes. The rest of the chamber with the small pieces of bands was filled with the same TBE buffer. The whole apparatus was filled with 1X TBE buffer that was not autoclaved up to the height of the 1X TBE in the gel compartment. The current was run at 200V and 4 °C. The electroelution process was monitored by periodic measurement of ODs every 30 min until the OD values leveled out or began to drop.

At the end of electroelution, 1/10th volume of 3M sodium acetate pH 5.2, and 3X absolute ethanol were added and either precipitated at −80 °C for 1 hour or -20 °C overnight. The DNA template or primer was centrifuged at 12,000 rpm for 30 min at 4 °C, the supernatant was poured out and the pellet air-dried. The dry pellet was dissolved in water after which the concentration was determined from OD measurements and appropriate conversion factor as indicated in Table 5.1.

5.4.2 Transcription

Transcription was done using a single-stranded DNA template from section 5.4.1. Prior to transcription, the primer also from section 5.4.1 was annealed to the template to produce a double stranded promoter region and transcription initiation site. In the annealing process, 8 µM of DNA template and 8 µM of the primer were mixed in a 1:1 ratio, then heated at 60 °C for 1 minute and cooled quickly on an ice bath. The transcription mixture contained 250 µl of 20X transcription buffer (20 mM Tris-HCl pH 8.1, 20 mM Spermidine, 100 mM dithiothreitol (DTT) and 0.2% triton X-100), 250 µl of 80 mM each Deoxynucleotide triphosphates (dNTPs), 250 µl of 80 mM of Guanosine 5’-Monophosphate (GMP), 140 µl of 1 M MgCl2, 500 µl of 8 µM of previously annealed 1:1 mixture of DNA template and primer, 1,000 µl of 400 mg/ml polyethylene glycol and
250 µl of T7 polymerase. The volume was adjusted to 5 ml with double distilled water and the mixture incubated at 37 °C for two hours. The transcription was stopped by addition of 1 ml of 0.25 M EDTA.

5.4.3 RNA extraction

The 5 ml of newly transcribed RNA was extracted using phenol extraction, in which 3 ml of phenol was added followed by 3 ml of chloroform/isoamyl alcohol (24:1 mixture). The sample was mixed and centrifuged at 2,300 rpm for 1.5 min at room temperature. The supernatant (top) layer was collected in an opaque Teflon tube and 2 ml of 100 mM Tris added to the bottom layer. The RNA was then centrifuged at the same conditions and supernatant collected and added to the previous one. One-tenth volume of 3 M Sodium acetate pH 5.2 (800 µl) and three times volume (24 ml) of chilled absolute ethanol was added and either precipitated at -80 °C for one hour or at -20 °C overnight. The precipitated RNA was centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatant was removed and the pellet air-dried and dissolved in 150 µl of double distilled water.

5.4.4 Purification of RNA

An equal volume of 2X gel loading buffer without dye (see appendix A) was added to the RNA prior to loading on a preheated 20% denaturing acrylamide (19:1 acrylamide: bis acrylamide) gel. The RNA was purified at 30 Watts and 1100 Volts for 10 hours in the case of the microhelix RNA and 12 hours for the antiterminator RNA due to the difference in the number of nucleotides. At the end of the purification, the position of the RNA bands were identified by UV shadowing. The bands were excised
using a sterile scalpel and electroeluted immediately or kept at -20 °C in a graduated centrifuge tube.

**5.4.5 Electroelution of RNA**

Electroelution of RNA was conducted as in section 5.4.1 above. After electroelution, RNA was ethanol precipitated at either -80 °C for 1 hour or at -20 °C overnight. It was then centrifuged, and the pellet air-dried and dissolved in about 100 ml of double distilled water.

**5.4.6 Lyophilizing RNA**

The dissolved RNA was lyophilized if necessary using the Flexi-dry system from Divetic Equipment Company for at least 6 hours. The RNA was first frozen in 1.5 ml tubes on dry ice. The tubes were covered with a Para film in order to prevent the RNA from being sucked out. Perforations were made on the Parafilm so as the water could be lyophilized away. After lyophilizing, the RNA was resuspended in approximately 50 µl of double distilled water. This was then followed by dialysis of the RNA.

**5.4.7 Dialysis**

Prior to dialysis of RNA the pre-dialysis ODs of the RNA sample were measured and recorded. The dialysis was run against 2 liters of autoclaved 5 mM sodium phosphate buffer pH 6.5, 0.05 mM EDTA or 10 mM sodium phosphate buffer pH 6.5, 0.01mM EDTA using the 7 Spectra/Por® dialysis membrane molecular weight 1,000 Daltons obtained from Spectra Chemical Laboratories Inc. The 5 mM buffer was used if the RNA was required to be very concentrated. The first liter of buffer was run for 8 hours then the remaining one run for about 12 hours. The dialysis membrane was
first rinsed several times with distilled water, making sure that it did not dry out before assembling it in the dialysis apparatus.

For RNA dialyzed in 10 mM buffer, the RNA was collected from the dialysis chamber at the end of dialysis. While for the 5 mM buffer, the volume after dialysis was determined and recorded. The RNA was lyophilized as previously described and half the after dialysis volume added to ensure the concentration of sodium phosphate buffer was now 10 mM. The UV readings were determined as described in section 5.1. The quality of the RNA was determined by monitoring the $A_{260}/A_{280}$ ratio (Sambrook et al., 1989). An acceptable ratio was between 1.7 and 2.0. The concentration was calculated from ODs as previously described. All the RNA was kept at -20 °C until use.

### 5.5 Preparation of fluorescently labeled RNAs

The 5′-Rhodamine fluorescently labeled RNAs and 2-aminopurine internally labeled RNAs were ordered from Dharmacon in a lyophilized, HPLC purified, deprotected and desalted condition. This was dissolved in 50 µl of double distilled water and dialyzed against 10 mM sodium phosphate buffer as previously described except that the dialysis chamber was covered with aluminum foil so as to protect against photo bleaching of the fluorophore. The UV readings were determined as previously described in section 5.1. For the rhodamine labeled RNAs the program used for UV readings was FIXλ. Rhd-RNA and the corrected values of $A_{260}$ and $A_{280}$ were determined by subtracting the $A_{554}$ from each $A_{260}$ and $A_{280}$ so as to account for absorbance of the fluorophore (Walter et al., 1996). The concentration was calculated using the extinction coefficients obtained from Dharmacon as indicated in table 5.2.
5.6 Preparation of tRNA

5.6.1 PCR for plasmid preparation

A PCR was carried out on a blunt double-stranded DNA plasmid that had been previously cloned (Fauzi 2005). The reaction mixture contained 1X Stratagene® cloned Pfu buffer, 0.2 µM dNTPs, 0.4 µM of each 5′-primer tRNA^Tyr-UCGA (5′-GAT AATACGACTCAGTGGAGGGTAGCG-3′), 3′-primer tRNA^Tyr-UCGA (5′-TGGAGGAGGGGGCAG-3′), 1µl of plasmid containing tRNA template and 2 µl Stratagene® Pfu Turbo polymerase in a total volume of 100 µl reaction. The underlined sequence on the 5′-tRNA primer is the T7 polymerase promoter region. Using the PTC-100™ programmable thermal controller by MJ research INC, the PCR reaction was initially denatured at 95 °C for 1 min then run through 25 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min and a final extension of 72 °C for 10 min.

The PCR product was confirmed on a 3% agarose gel stained with 0.5 µg/µl of ethidium bromide in 0.5X TBE and band size visualized using a UV transilluminator from NucleoTech. The size of the PCR product was measured with a 10 bp ladder obtained from Promega. The PCR product was then cleaned with ExoSAP IT from USB® (2 µl per 5 ml PCR reaction) to remove the excess dNTPs that would interfere with subsequent reactions. It was then incubated at 37 °C for 15 min followed by enzyme inactivation at 80 °C for 15 min (Fauzi et al., 2005)

5.6.2 Ligation of the PCR product

The PCR product was ligated into PCR® Blunt vector obtained from Invitrogen. The ligation reaction contained 1 µl of the vector as supplied by the vendor, 1 µl of 10 X Ligation buffer with ATP (0.5 M Tris-HCl (pH 7.8), 0.1 M MgCl₂, 0.1 M DTT, 10 mM
ATP), 1µl of T4 DNA ligase obtained from Ambion® and 3 µl of the PCR product from section 5.6.1 in a final volume of 10 µl. The mixture was incubated at 16 ºC for 1 hour. Then kept on ice in preparation of transformation (Sambrook et al., 1989)

5.6.3 Transformation

The ligation product was transformed using the One Shot® Top 10 chemically competent cells from Invitrogen. The SOC media supplied with the One Shot® Top 10 cloning kit was first warmed to 37 ºC. The vials with the competent cells were thawed on ice before addition of 2 µl of the ligation reaction from section 5.6.2. The vials were swirled gently then incubated at 42 ºC for exactly 45 sec without shaking and immediately transferred on the ice for 2 min. Prewarmed SOC media (250 µl) was added to each vial, the vials were placed on microfuge rack and shaken on a Beckman Coulter JS-HS centrifuge at 37 ºC, 225 rpm for 1 hour. After shaking the vials were placed on ice until ready for plating.

5.6.4 Preparation LB broth and plates

The Luria-Bertani (LB) media containing 1.0% Tryptone, 0.5% Yeast extract and 1.0% NaCl (Sambrook, 1989) was prepared by mixing 5g of EZ mix tryptone, 2.5g of EZ mix yeast extract and 5g of NaCl (all obtained from Sigma Aldrich). The mixture was adjusted with distilled water to 500 ml and pH adjusted to 7 with 12 M NaOH. In a half of this media (250 ml), 3.75 agar was added and autoclaved for 25 min. The remaining media was autoclaved for 25 min and kept at 4 ºC for future use. After autoclaving, the media was cooled to ~ 55 ºC before adding 12.5 mg of kanamycin. The media was poured into Fisher Brand 10 cm sterile Polystyrene plates in the hood.
and allowed to set. After setting, the plates were covered, inverted and stored at 4 °C until use.

5.6.5 Plating
The plates from section 5.6.4 were pre warmed at 37 °C before plating. Plating was done in the hood so as to ensure sterile conditions and minimize contamination. A flame was present at all times during plating. The cells from transformation step were spread using a metallic spreader. The spreader was dipped in absolute ethanol and dried on the flame before being used for spreading. It was also necessary to cool the spreader first after passing it on the flame so as to avoid killing the cells. Two plates were used for each vial and two different volumes 10 µl and 30 µl were pipetted on the plates. For smaller volumes, 20 µl of SOC medium was added so as to ensure even spreading. The plates were covered, inverted and incubated overnight at 37 °C.

5.6.6 Selection of colonies
Individual colonies from the overnight incubation were picked and touched on a replicate plate labeled with Petri Stickers obtained from Diversified Biotech. The labeling of the stickers on the plates was necessary so as to be able to locate a particular colony for future reference. The initial plate (duplicate plate) was covered with Parafilm and stored at 4 °C. Meanwhile, 2 ml LB media from section 5.6.4, containing 50 mg/ml of kanamycin was transferred to sterile Gamma irradiated falcon tubes obtained from Becton Dickinson and inoculated with the cells from the replicate plate. The culture was then shaken at 37 °C using a Lab-line® incubator shaker at a speed of 225 rpm overnight making sure that the caps are loosely closed.
5.6.7 Extraction of the plasmid

A cloudy broth from the overnight culture was centrifuged for 15 min at 4 °C and the plasmids extracted following the bench protocol described in the Qiagen Prep Spin Miniprep Kit using a vacuum Manifold. The size of the extracted plasmid was confirmed on a 0.7% agarose gel in 1X TBE buffer by running at 125 volts for 1 hour. The band size was measured using the 1 Kb ladder obtained from Promega. The plasmid was sent for sequencing at Ohio University Genomic Facility and the correct sequence confirmed using Invitrogen Vector NTI program version 10.0.1.

5.6.8 Transcription of tRNA

A PCR reaction was first performed as previously in section 5.6.1 and 6 µl of the template was used in the transcription reaction. Each transcription reaction contained 4 µl of 10X T7 flash reaction buffer (appendix), 3.6 µl of 100 mM of ATP, CTP GTP UTP, 4 ul of DTT and 4 µl of the T7 Flash enzyme solution all from the Ampliscribe™ T7 flash kit obtained from Epicenter Biotechnologies in a total volume of 40 µl. The mixture was incubated at 42 °C for 1.5 hours before addition of 2 µl of RNase free DNAse -1 also from the Ampliscribe™ T& Flash kit and incubated for 15 min at 37 °C. Equal volume of 2X loading buffer was added heated at 90 °C for 1.5 min and loaded on a Prewarmed 20% denaturing polyacrylamide (19:1 acrylamide: bis acrylamide) gel. The gel was run for 6 hours at 1750 V and 30 Watts. The bands were UV shadowed and excised out as previously described in section 5.4.3. Elution buffer (appendix) 500 µl was added and shaken for 4 hours on a Titech Micromixer-E36 followed by ethanol precipitation. The tRNA was then centrifuged using an Eppendorf Centrifuge 5415D at 13,200 rpm at 4 °C for 1 hour, washed twice with 80% ethanol and dried using a DNA 120 Speed Vac® obtained from Thermo Savant®. The dried tRNA
was resuspended in RNase free Ultra pure MB grade water and filtered using Ultra Free®-MC 0.22 μM low binding Durapore® Membrane from Amicon Bio-separations. It was then lyophilized and dialyzed in either 5 mM or 10 mM dialysis buffer. Following dialysis, the tRNA was collected from the dialysis chamber and volume recorded. The tRNA that was dialyzed in 5 mM buffer was lyophilized for a minimum of 6 hours and half volume of RNase free water added. The UV readings were taken and concentration calculated using the appropriate conversion factor as previously described in section 5.1, then kept at -20 °C until use.

5.7 Prediction of RNA secondary structure

The secondary structures of the antiterminator RNAs and microhelix models were predicted using, a free energy minimization with energy parameters based on the nearest neighbor model (Zuker, 2003). The primary sequence was first determined based on the B. Subtilis TyrS antiterminator model AM1A and microhelix tRNA models (Gerdeman 2002). The loop regions were substituted with various tetraloops belonging to the GNRA and UNCG family (Tuerk et al., 1988a; Woese et al., 1990). The resultant secondary structure was predicted using Mfold software and parameters set to a window size of 2, a 20 maximum number of structures and 50% sub optimality, The secondary structures that folded properly and had the minimum energy were selected for further investigation while making sure that the next suboptimal structure was at least 1 Kcal higher than the optimal structure so as to prevent mixtures in solution.

5.8 Circular dichroism (CD)

The CD technique was employed to monitor the characteristics of the antiterminators and the microhelix models as result of changes in salt concentration,
loop region and pH changes. The spectra were collected using a Jasco 700 spectrophotometer in a quartz cuvette with a path length of 1mm. All samples were analyzed in 200 µl final volume at 4 ºC. RNA was first allowed to equilibrate to ambient temperature then heated at 90 ºC and cooled slowly to room temperature before initiating any scan.

5.8.1 Effect of loop stability on CD of antiterminators

RNA was first dialyzed in 10 mM sodium phosphate buffer pH 6.5, 0.01 mM EDTA. A final concentration of 10 µM RNA was scanned in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 0.01 EDTA and either 5 mM or 15 mM MgCl₂. The scans were started at 320 nm and stopped at 200 nm at a rate of 20 nm/min, response of 8 sec and a collection of 3 scans was averaged for each spectra. Before analyzing the data, a blank scan with buffer alone of each antiterminator was subtracted from scans containing RNA. The data was exported as text files and analyzed in excel. Molar ellipticity was calculated according to the equation:

\[ \theta = \frac{\Theta}{32,980 \times C \times L \times N} \]

Where \( \theta \) = experimental CD amplitude in mdeg, \( C \) = RNA concentration in mol/L, \( L \) = cuvette path length in cm (0.1) and \( N \) = Number of nucleotides in RNA (Johnson, 1996)

5.8.2 Effect of pH on CD of antiterminator AM1A and C11U

The pHs studied were 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0. In order to ensure good buffering conditions, various buffers were used. This was done in order to ensure that the pKa of the buffer being used was close to the required pH. For instance the Mes buffer (pKa 6.1) was used for pH 5.0, 5.5 and 6.0. Sodium phosphate buffer (pKa
(pKa 8.06) was used for pH 7.5 and 8.0. The CD scans were performed in 50 mM concentrations of the various appropriate buffers, 50 mM NaCl, 15 mM MgCl₂ and 0.01 mM EDTA. The rest of the parameters were as in section 5.8.1 above.

5.8.3 Effect of pH on microhelix-UCCA and its complex with AM1A

The study of effect of pH on microhelix was carried out as in section 5.5.2 above. For the complex with AM1A only three pHs were investigated. That is pH 5.0, pH 6.5 and pH 7.5. For the complex experiment, each RNA was renatured separately then cooled and combined. A final concentration of 10 µM complex RNA was analyzed, (i.e 10 µM of AM1A plus 10 µM mhUCCA). The mathematical summation was calculated by adding the experimental CD raw data of each RNA and analyzing the molar CD of the total complex. In each case, the molar CD was calculated from the equation in section 5.8.1, where the RNA concentration used was 10 µM and the total number of nucleotides used in the calculation was 51 (i.e 29 nucleotide for AM1a and 22 nucleotides for mhUCCA). The rest of the parameters were as in section 5.8.2 above.

5.9 Enzymatic probing

Enzymatic probing was used to investigate the secondary structure of different antiterminators and microhelices depending on the accessibility of the enzyme to specific nucleotides. The different antiterminators studied were AM1A, C11U, AM2, AM3, AM4, AM5 and AM6. The different microhelices were mhUCCA, mh1, mh2, mh3 and mh4. Three different RNases were employed in order to cleave the RNA molecules depending on their specificity and accessibility. The single stranded specific
RNase T1 and A was used to monitor unpaired Gs and Cs/Us respectively. While the double stranded or stacking specific RNase V1 was used to monitor paired and stacked regions.

5.9.1 Labeling of RNA

5.9.1.1 Alkaline phosphatase reaction

The various antiterminator and microhelix RNAs were labeled at the 5′end with $\gamma$-P$^{32}$ ATP using a 5′ end Labeling Kinase Max kit from Ambion. All procedures involving $\gamma$-P$^{32}$ were confined in the radioactive material section. The $\gamma$ phosphate was first cleaved using calf intestine phosphatase in a reaction mixture containing 2.5 $\mu$M of RNA, 1 X dephosphorylation buffer, 1 $\mu$l of calf intestine phosphatase and nuclease free water up to a volume of 10 $\mu$l. The reaction was incubated at 37 °C for one hour in a 0.2 ml thin wall RNase and DNAse free tubes of optimum research grade in a PTC-100TM programmable thermal controller from MJ Research, INC.

After this incubation, 10 $\mu$l of Phosphatase Removal reagent (PRR) was added to remove the phosphatase enzyme. This was incubated for three min at room temperature with occasional flicking. The tubes were centrifuged briefly in a microfuge (~15 sec of ramping up to top speed) to pellet the PRR. The supernatant containing the alkaline phosphatase reaction was transferred to a fresh microfuge tube and preceded to the next reaction.

5.9.1.2 Kinase reaction

About 15 $\mu$l of the above reaction was used in a reaction containing 2 $\mu$l of [\gamma-^{32}P] ATP, 2 $\mu$l of 10 X kinase buffer and 1 $\mu$l of T4 polynucleotide kinase making a total
reaction volume of 20 μl. The reaction was incubated at 37 °C for 1 hour as the above alkaline phosphatase reaction.

5.9.2 Evaluation of the labeled reaction

In order to evaluate the radio labeled reactions, the RNA was first purified on a preheated 20% denaturing polyacrylamide (19:1 acrylamide: bis acrylamide) gel for 3 hours. The gels were covered with fisher brand polyvinyl chloride paper and luminescent paper attached as a guide for correct orientation of the bands. This was then transferred to an intensifying cassette for film exposure. A Kodak intensifying film was placed on the gel for 1.5 min and developed by immersing in the Kodak GBX developer and replenisher. The film was rinsed several times in distilled water then fixed in a Kodak GBX fixer and replenisher for another 1.5 min, rinsed a gain and hang up to dry. Excision of the band from the gel was followed after correct orientation of the gel on the film using the alignment of the luminescent paper. The bands were kept at -20 °C in a 1.5 ml centrifuge tube until use.

5.9.3 Elution and precipitation of the RNA

The labeled RNA was eluted from the bands by shaking for 4 hours in 500 μl of elution buffer (Appendix). The supernatant was taken out into a fresh 1.5 ml microfuge tube and 3X chilled absolute ethanol was added and precipitated at either -80 °C for 1 hour or at -20 °C overnight. The precipitated RNA was centrifuged at 4 °C for 60 min at 132,000 rpm. The supernatant was removed and the pellet washed with 80% Ethanol and dried in a speed vacuum. The tubes were covered with a Para film and perforations inserted on the Para film to prevent loosing the RNA. RNA was reconstituted by dissolving in 10 μl of 10 mM Sodium phosphate buffer pH 6.5, 0.01
mM EDTA. At the end of this step, the labeled RNA was about 300,000 – 500,000 counts per meter (CPM) and was ready for the probing experiments.

5.9.4 RNase T1, A and V1 Probing

Probing was proceeded by incubating 0.25 μM of the labeled RNA in a final concentration of 1X structure buffer (appendix), 0.3 μg/μl of yeast RNA and 1 μl of 1/10th RNase A and T1 or 1/20th of RNase V1. For microhelix probing, the RNase V1 used was 1/100th. All the reactions were incubated at room temperature. An alkaline hydrolysis reaction was also included in order to measure the band size. This was obtained by incubating 0.25 μM of the labeled RNA in 9 μl of alkaline hydrolysis buffer at 90 °C for 15 min. While its control was prepared by incubating the same concentration of the labeled RNA in 10 mM sodium phosphate buffer under the above conditions. All reactions were stopped by adding equal volume of 2X gel-loading buffer with dye (appendix). The samples were loaded on 20% denaturing polyacrylamide (19:1 acrylamide: bis acrylamide) gel after heating for 1.5 min at 90°C. The gel was run at 30 watts for 3 hours after which it was taken off and covered with Fisher brand polyvinyl chloride paper and transferred to an intensifying cassette for film exposure.

5.9.5 Exposing of the intensifying films

An intensifying Kodak film was placed on the above gel in the dark after which the cassette was taken to an -80 °C freezer and left to expose between 1 and 2 hours. The cassette was taken from the freezer at allowed to equilibrate to room temperature before taking out the film. The film was then developed in the dark room for 1.5 min by immersing in the Kodak GBX developer and replenisher. This was followed by several
times of rinsing in distilled water then fixed in a Kodak GBX fixer and replenisher for another 1.5 min, rinsed a gain and hang up to dry.

### 5.9.6 Analysis of the bands

The exposed films from above were scanned using EPSON perfection 3490 photo scanner. The scanned films were adjusted using ADOBE Photoshop CS2 software and saved as TIFF files. Bands were then analyzed using a gel document in which the entire lane was analyzed and each band normalized.

### 5.10 Determination of thermal stability of different RNAs using UV monitored thermal denaturation

The stability of different antiterminator and microhelix RNA was determined experimentally using UV absorbance melting curves (Puglisi and Tinoco, 1989). The melting profiles were investigated using a Beckman Coulter Counter® 640 spectrophotometer equipped with a temperature controller. The melts were collected between 2 °C to 95 °C in a 1 cm micro cell cuvette equipped with a fitted stopper. The RNA was first dialyzed in 10 mM Sodium phosphate buffer pH 6.5 and 0.01 mm EDTA. The RNA was allowed to equilibrate to room temperature then heated at 90 °C for 1.5 min and allowed to cool slowly to room temperature for 10 min. The required volume was added to a1.5 ml microfuge tubes so as to have a final concentration of 1.5 µM of RNA in 10 mM sodium phosphate buffer pH 6.5, 0.01 mM EDTA and adjusted with water to give a final volume of 320 µl. The blank tube was also prepared with just buffers only and no RNA. Each tube was degassed with a stream of argon gas for ~30 sec and carefully transferred to the cuvettes without introducing air bubbles. A total of 6 sample cells could be analyzed in each melt cycle. The cuvettes were loaded on the cell chamber and closed followed by inserting the argon inlet tube. Prior to each
melt cycle, it was necessary to purge the entire chamber and the cell chamber with argon gas so as to prevent moisture condensation on the cells. Argon was purged to the entire chamber for 15 min before blanking then purged again for another 15 min after clicking read samples on the spectrophotometer. At the end of this second 15 min purging, the argon gas was introduced to the sample compartment via an inlet tube and purged for 5 min before initiating the melt cycle. There was a hold period of 10 min before the actual melt began. Absorbance values were monitored at 260 nm at a scan rate of 0.5 °C and ramp rate of 1 °C/min.

For the investigation of magnesium dependence stability of different antiterminators. All experimental parameters were conducted as above except that the RNA was analyzed in 10 mM sodium phosphate buffer pH 6.5, 90 mM NaCl, 0.01 mM EDTA and either 5 mM or 15 mM MgCl₂. All the UV melt data was acquired and processed using the Beckman Coulter Data Capture in order to transport the raw data to Microsoft Excel 2004. The data was analyzed using OD Deriv (Draper and Gluick, 1995) then the first derivative of absorbance/temperature (dA/dT) was plotted against temperature.

5.11 Fluorescence based binding assays

5.11.1 Multiwell fluorescence studies

The experiments were performed on a Flex Station Molecular Device. Fluorescently labeled RNAs were used to investigate various aspects of antiterminator RNA due to changes in the loop region, salt concentration, pH and initial screening of different compounds. The fluorophores used were rhodamine and Fluorescein. Rhodamine was attached at the 5’end of the antiterminator RNAs while Fluorescein was attached at the 3’end. In Fluorescence Resonance Energy Transfer (FRET) the
Fluorescein was attached at the 3’end while the rhodamine was attached at U18 of AM1A. All the experiments were performed on a 96 well UV star flat bottom plates from Griener Bione. A total reaction volume of 100 ul was loaded per well. The final concentration of fluorescently labeled RNA in all assays was 100 nM. Reagents were first prepared on a reagent plate (See Table 5.3 as an example) then pipetted onto the final read plate (Example Table 5.4). All the RNAs were heated at 90 °C for 1.5 min and cooled to room temperature for at least 10 min before pippeting on the plates.

For the experiments involving rhodamine labeled RNA, the fluorophore was excited at 540 nm, with a cut off at 570 nm and the spectrum collected between 540-650 nm with a step resolution of 5 nm. For Fluorescein labeled RNA, the fluorophore was excited at 467 nm with a cut off of 495 nm and emission spectrum collected between 505 and 560 nm at a step resolution of 5 nm. While for FRET the fluorophore was excited at 490 nm with a cut off filter of 515 nm and the spectrum collected between 515 and 640 nm at a step resolution of 5 nm. All the multi well fluorescence assay plates were read after 10 min, 60 min and 120 min incubation time at 25 °C. The binding curve and binding affinities were obtained by calculating the F_{rel} at 585 nm from the following equation:

\[
F_{rel} = \frac{|F-F_0|}{F_0}
\]

Where F is the fluorescence intensity at 585 nm of the fluorescently labeled RNA/ligand complex at each ligand concentration and F_0 is the fluorescence intensity of at 585 nm of the fluorescently labeled RNA without a ligand. The F_{rel} was plotted versus the concentration of the ligand (tRNA-UCCA, microhelices or compounds) using
the Prism (Graph Pad) in which the one site binding was compared to the two-site binding.

5.11.2 Binding assays for tRNA-UCCA and microhelix UCCA to Antiterminators: Effect of metal ions

The binding assays were performed in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 0.01 mM EDTA and either 0, 5 mM or 15 mM MgCl₂ was used for most of these fluorescence experiments. For the NaCl study, NaCl concentration used ranged from 0 to 200 mM while MgCl₂ was eliminated completely. For the CaCl₂ study up to 2.0 µM of Ca²⁺ was substituted for Mg²⁺. While for cobalt hexamine (III) chloride study, up to 2 mM of Co³⁺ was substituted in the place of Mg²⁺. The binding mixture contained 100 nM of 5'-Rhd antiterminator and 25 µl of either tRNA or microhelix-UCCAs in a serial dilution. The concentration of tRNA-UCCA ranged from 0 to 2.6 µM While the microhelix-UCCA concentration ranged from 0 to 312 µM. Control wells containing buffer only and the 5'-Rhd labeled RNA were also included on the plate reader.
Table 5.3  An example of a reagent plate for tRNA-UCCA binding to 5′-Rhd-AM1A. The numbers indicate the columns while the letters shows the rows. The contents of each column are shown. The concentrations of tRNA-UCCA in serial dilution are shown in the last two columns.

<table>
<thead>
<tr>
<th></th>
<th>10 mM Naphos buffer pH 6.5, 0.01 mM EDTA (230µl each well)</th>
<th>5′Rhd-AM1A stock in 10mM NPB, 0.01mM EDTA pH 6.5 (110ul each well)</th>
<th>5′Rhd-AM1A stock in 10mM NPB, 0.01mM EDTA pH 6.5 (110ul each well)</th>
<th>tRNA-UCCA stock in 10mM Naphos, 0.01 mM EDTA pH 6.5, 134µl +110µl Naphos</th>
<th>tRNA-UCCA stock in 10mM Naphos, 0.01 mM EDTA pH 6.5, 134µl +110µl Naphos</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Buffer only</td>
<td>400 nM 5′Rhd-AM1A</td>
<td>400 nM 5′Rhd-AM1A</td>
<td>10.4 µl tRNA-UCCA</td>
<td>10.4 µl tRNA-UCCA</td>
</tr>
<tr>
<td>B</td>
<td>Buffer only</td>
<td>400 nM 5′Rhd-AM1A</td>
<td>400 nM 5′Rhd-AM1A</td>
<td>5.76 µM tRNA-UCCA</td>
<td>5.76 µM tRNA-UCCA</td>
</tr>
<tr>
<td>C</td>
<td>Buffer only</td>
<td>400 nM 5′Rhd-AM1A</td>
<td>400 nM 5′Rhd-AM1A</td>
<td>3.2 µM tRNA-UCCA</td>
<td>3.2 µM tRNA-UCCA</td>
</tr>
<tr>
<td>D</td>
<td>Buffer only</td>
<td>400 nM 5′Rhd-AM1A</td>
<td>400 nM 5′Rhd-AM1A</td>
<td>1.76 µM tRNA-UCCA</td>
<td>1.76 µM tRNA-UCCA</td>
</tr>
<tr>
<td>E</td>
<td>Buffer only</td>
<td>400 nM 5′Rhd-AM1A</td>
<td>400 nM 5′Rhd-AM1A</td>
<td>0.96 µM tRNA-UCCA</td>
<td>0.96 µM tRNA-UCCA</td>
</tr>
<tr>
<td>F</td>
<td>Buffer only</td>
<td>400 nM 5′Rhd-AM1A</td>
<td>400 nM 5′Rhd-AM1A</td>
<td>0.52 µM tRNA-UCCA</td>
<td>0.52 µM tRNA-UCCA</td>
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<tr>
<td>G</td>
<td>Buffer only</td>
<td>400 nM 5′Rhd-AM1A</td>
<td>400 nM 5′Rhd-AM1A</td>
<td>0.28 µM tRNA-UCCA</td>
<td>0.28 µM tRNA-UCCA</td>
</tr>
<tr>
<td>H</td>
<td>Buffer only</td>
<td>400 nM 5′Rhd-AM1A</td>
<td>400 nM 5′Rhd-AM1A</td>
<td>0.0 µM tRNA-UCCA</td>
<td>0.0 µM tRNA-UCCA</td>
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</tbody>
</table>
Table 5.4 An example of a read plate for tRNA-UCCA binding to 5’-Rhd-AM1A. This figure shows final experiment conditions after all manual and automated pipetting.

<table>
<thead>
<tr>
<th></th>
<th>OmM MgCl₂</th>
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<th></th>
<th>5mM MgCl₂</th>
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<th>15mM MgCl₂</th>
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<tr>
<td>A</td>
<td>100mM buffer</td>
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5.11.3 Binding assays for tRNA-UCCA to 5′-Rhd-AM1A and 5′ Rhd-C11U in different pH

Each reaction well contained 100 nM of 5′-Rhd-AM1A or 5′-Rhd-C11U, 50 mM of various buffers of pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, 50 mM NaCl, 0.01 mM EDTA, 15 mM MgCl₂ and tRNA-UCCA ranging from 0 - 2.6 µM in serial dilution. The different buffers prepared for the various pH were as follows; Mes buffer (pKa 6.1) was used for pH 5.0, 5.5 and 6.0. Sodium phosphate buffer (pKa 7.0) was used for pH 6.5 and 7.0 while Trizma buffer (pKa 8.06) was used for pH 7.5 and 8.0.

5.11.4 Initial screening of antiterminators

For the initial screening experiments, the 5′-Rhd RNAs were incubated in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, and 5 mM MgCl₂ and 0.01 mM EDTA. The different compounds were added up to a final concentration of 10 µM. Tables 5.5 and 5.6 shows an example of reagent plate and read plate set up respectively. The final read plate was covered with aluminum foil and rocked for 5 min on a Lab-Line Maxi Rotator at maximum speed before loading on the Flex Station Device. The normalized percentage was calculated according to the following equation:

\[
\left( \frac{F}{F_0} \right) - 1 \times 100
\]

Whereby F is the fluorescence at 585 nm of the 5′-Rhd RNAs in the presence of the compound and \( F_0 \) is the fluorescence at 585 of the 5′-Rhd RNAs without the compounds.
Table 5.5: An example of a reagent plate for initial screening of different compounds binding to 5'-Rhd-AM2 and 5'-Rhd-GlyQS. The numbers indicate the columns while the letters shows the rows. The contents of each column are shown. Each well from columns 4-8 represents 40 µM of different compounds.

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<td>A</td>
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<td>DMSO/H2O</td>
<td>400 nM 3'Fl-AM1A-Rhd</td>
<td>400 nM 3'Fl-C11U-Rhd</td>
<td>40µM GHB-52</td>
<td>40µM IMB-39</td>
<td>40µM COB-30</td>
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Table 5.6 An example of a read plate for initial screening of different compounds binding to 5'-Rhd-AM2 and 5'-Rhd-GlyQS. This figure shows final experiment conditions after all manual and automated pipetting.

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100nM 5'-Rhd-AM2
DMSO/H2O

100nM 5'-Rhd-GlyQ
DMSO/H2O

100nM 5'-Rhd-GlyQ
DMSO/H2O

100nM 5'-Rhd-GlyQ
DMSO/H2O

100nM 5'-Rhd-GlyQ
DMSO/H2O

100nM 5'-Rhd-GlyQ
DMSO/H2O

100nM 5'-Rhd-GlyQ
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100nM 5'-Rhd-GlyQ
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100nM 5'-Rhd-GlyQ
DMSO/H2O

100nM 5'-Rhd-GlyQ
DMSO/H2O
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Table 5.6: continued
5.11.5 Steady state single well fluorescence studies

The 2-AP studies were done in a single well in a 600 μl quartz cuvette using the Fluoromax-3 Jobin Yvon Horiba instrument equipped with a temperature control. The RNAs were first heated at 90 °C for 1.5 min and cooled for at least 10 min at room temperature. Each scan of 100 nM 2-aminopurine internally labeled antiterminator RNAs 9-AP-AM1A, 9-AP-AM2 and 9-AP-GlyQS was performed in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 0.01 mM EDTA and either 5 mM or 15 mm MgCl₂ at 20 °C. The buffer alone 398 μl was allowed to equilibrate to 20 °C before taking the first scan. This was followed by addition of 2 μl of 21 μM 9-AP antiterminators. Titrations of tRNA up to 10 additions each 2 μl were added and a scan collected each round. After every titration, the contents in the cuvette were mixed thoroughly using fisher brand sterile cuvette stirrers, and allowed to equilibrate for 5 min before taking any scan. The final concentration of tRNA-UCCA added was 15 μM. For the microhelix-UCCA the same titrations of 2 μl each were added up to 140 μM concentration. At the end of all the titrations, the final volume in the cuvette was 420 μl. The scans were excited at 310 nm starting at 330 nm and ending at 600 nm at a rate of 1 second. The slit width for both excitation and emission spectra were set to 5 nm. The background subtractions were done by subtracting the scan of buffer alone from each titration scan containing RNA. Titrations of 2 μl sodium phosphate buffer were added up to 20 μl to 9-AP-RNAs in buffer as a control.
REFERENCES


Bloomfield, V. A.; Crothers, D. M.; Tinoco, I. Nucleic acids structure, properties and functions, University of science books: Sausalito, CA, 2000; p 13-41.


Daly, T. J.; Rusche, J. R.; Maione, T. E.; Frankel, A. D. Circular-Dichroism Studies of the HIV-1 Rev Protein and Its Specific RNA-Binding Site. *Biochemistry* 1990, 29, 9791-9795.


Jack, K. D. Metal ion effects on the T box antiterminator RNA and complex formation with tRNA. Ohio University, Athens, 2007.


Yousef, M. R.; Grundy, F. J.; Henkin, T. M. Structural transitions induced by the interaction between tRNA(Gly) and the Bacillus subtilis glyQS T box leader RNA. *Journal of Molecular Biology* 2005, 349, 273-287.


APPENDIX A: REAGENT SOLUTIONS AND ABBREVIATIONS

NPB- Sodium phosphate buffer

10 X Tris borate EDTA (TBE) buffer
500 mM Tris-Borate, 10 mM EDTA

2X gel-loading buffer without dye
2 x TBE, 9 M urea and 1 mM EDTA

2X gel-loading buffer with dye
2x TBE, 9 M urea and 1 mM EDTA, 0.02% Bromophenol blue, 0.02% xylene cyanol

Binding buffer
50 mM Sodium phosphate pH 6.5, 50 mM NaCl, 5 mM MgCl₂ and 0.01 mM EDTA

Dialysis buffer
10 mM Sodium phosphate pH 6.5, 0.01 mM EDTA

20 X Transcription buffer
20 mM Tris-HCl, 20 mM spermidine, 100 mM DTT, and 0.2% Triton X-100

Elution buffer
0.5 M sodium acetate, pH 5.2, 1 mM EDTA

10X Structure buffer
10 mM Tris pH 7, 1 M KCl, 100 mM MgCl₂

Pfu Turbo polymerase
2.5 U/µl

Calf intestine phosphatase
1U/µl

T4 polynucleotide kinase
10U/µl

RNase T1
1U/µl

RNase A
1µg/ml

RNase V1
0.1U/µl
Figure B.1 The UV derivative plots of antiterminator structures with different loop substitutions; ■ AM1A, ○ AM2, ▲ C11U, ◇ AM3, ◼ AM4, □ AM5, △ AM6, and ▼ GlyQS, the UV melts were measured between 4 °C – 95 °C in 10 mM sodium phosphate buffer pH 6.5 and 0.01 mM EDTA.
Figure B.2 The UV derivative plots of antiterminator structures with different loop substitutions. ◆ AM1A, ● AM2, ▲ C11U, ◈ AM3, ○ AM4, □ AM5, △ AM6 and ▼ GlyQS. The UV melts were measured between 4 °C – 95 °C in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 5 mM MgCl₂ and 0.01 mM EDTA.
Figure B.3 The UV derivative plots of antiterminator structures with different loop substitutions. The UV melts were measured between 4 °C – 95 °C in 10 mM sodium phosphate buffer pH 6.5, 90 mM NaCl, 0.01 mM EDTA and A, 0 mM, B, 5 mM and C, 15 mM MgCl₂
Figure B.4 The effect of pH on CD of A, AM1A and B, C11U. The scans were measured in 50 mM buffers; Mes (pH 5.0, 5.5, 6.0), sodium phosphate (pH 6.5, 7.0) and trizma (pH 7.5, 8.0) containing 50 mM NaCl, 15 mM MgCl₂ and 0.01 mM EDTA.
Figure B.5 RNAse T1 cleavage pattern of antiterminators AM1A, C11U, AM2 and GlyQS
Figure B.6 RNAse T1 cleavage pattern of antiterminators AM3, AM4, AM5 and AM6
Figure B.7 Graphical representation of RNase T1 cleavage in the A2 helix
Figure B.8 RNAse A cleavage pattern of antiterminators AM1A, C11U, AM2 and GlyQS
Figure B.9 RNase A cleavage pattern of antiterminators AM3, AM4, AM5 and AM6
Figure B.10 Graphical representation of RNAse A cleavage in the A2 helix.
Figure B11 RNAse V1 cleavage pattern of antiterminators AM1A, C11U, AM2 and GlyQS
Figure B.12 RNAse V1 cleavage pattern of antiterminators AM3, AM4, AM5 and AM6
Figure B.13 The UV scans of different 5’-Rhd antiterminator RNAs. The scans collected between 220 nm and 600 nm with 100 nM concentrations of each RNA.
Figure B.14 The binding isotherms of tRNA-UCCA to 5′-Rhd-AM1A and 5′-Rhd-C11U in the presence of 2 µM CaCl₂. The binding studies were performed in 50 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 0.01 mM EDTA and 2 µM CaCl₂. The K_d for AM1A was 0.19±0.18 µM and an R² of 0.41. There was no conversion for the C11U graph.
Figure B.15 RNAse T1 cleavage pattern of microhelices mhUCCA, mh1, mh2, mh3 and mh4
Figure B.16 RNAse A cleavage pattern of microhelices mhUCCA, mh1, mh2, mh3 and mh4
Figure B.17 RNAse A cleavage pattern of microhelices mhUCCA, mh1, mh2, mh3 and mh4