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## **Nucleic Acids: Thermal Stability and Denaturation**

DNA melting, RNA melting, duplex stability, base pairing

Doktycz, Mitchel J.

Oak Ridge National Laboratory, USA

Nucleic acid structures can have different stabilities that depend on a variety of factors that include the nucleic acid sequence, the structure adopted, and the solution environment. Together, these factors influence the thermal stability of the complex.

### **Introduction**

The nucleic acids DNA and RNA are characterized by the ability of an individual molecular strand to specifically pair with a second strand using the intrinsic pairing capabilities of the nucleotide bases to form a double stranded, helical structure. The stability of the double stranded structure is critically important for many aspects of nucleic acid metabolism. Strand separation must occur for DNA replication, for DNA repair and for the transcription of DNA into RNA. Likewise, the annealing of complementary sequences is equally important. Local, double stranded regions are found in the functional classes of RNA molecules and play essential roles in the formation of structures that are required for the molecule's function. Additionally, the stability of nucleic acid hybrids is playing an integral role in biomedical diagnostic applications. The specificity of hybridization is at the core of many molecular biology techniques including the polymerase chain reaction (PCR), DNA sequencing, Southern blotting, and emerging applications such as microarray technology.

The stability of the hybrid formed between the two strands can be assessed by several different biophysical techniques. One common approach is the use of spectrophotometric methods. Nucleic acids absorb strongly in the uv region (around 260 nm) and this absorbance increases upon the disassociation of the strands. This hyperchromicity results from the unstacking of the nucleotide bases. Typically, experiments monitor the absorbance as a function of temperature to provide a "melting curve" as shown in Figure 1. The midpoint of the transition is referred to as the melting temperature, or  $T_m$ , and is indicative of the thermal stability of the duplex or structure of interest. This transition is referred to as melting, due to its abrupt, cooperative nature. Other descriptions include denaturation, disassociation, or helix-coil transition. The reverse reaction is often termed hybridization, annealing, association, or duplex formation. The experimental melting, or annealing, curve can be analyzed by either van't Hoff (Marky and Breslauer, 1987) or statistical thermodynamic methods to provide thermodynamic information. Many factors can influence the melting temperature of nucleic acids, most importantly is the sequence

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itself, its length and fidelity of pairing. The sequence can also dictate the molecule's ability to form alternate structures. Extrinsic conditions, such as the salt concentration, pH, and presence of non-aqueous solvents can also greatly influence the thermal stability of nucleic acids. A few of the important features will be discussed below.

## Effects of Base Pairing

A central structural feature of double stranded nucleic acids is the interstrand hydrogen bonds that form between the A-T and G-C bases. This interaction stabilizes the structure against disassociating and provides for the hybridization specificity. Biophysical studies in the 1960's clearly established that duplex stability increased with the fraction of base pairs that are G or C. These early studies were performed on long natural DNA or RNA duplexes of varying GC content. This stability dependence is seemingly reconciled by the number of interstrand hydrogen bonds that are formed. G-C pairs form three hydrogen bonds while A-T base pairs form only two, as seen in Figure 2. Certainly other forces contribute to duplex stability including interactions with the solvent, the hydrophobic interactions between the bases, and the destabilizing, repulsive forces of the charged phosphate backbone. Collectively, base pair formation is enthalpically favorable, summing up to  $\sim -8.5$  kcal $\cdot$ mol A-T base pair $^{-1}$  and  $\sim -9.4$  kcal $\cdot$ mol G-C base pair $^{-1}$ . However, base pair formation is not entropically favorable and carries a penalty of  $\sim -24.85$  cal $\cdot$ mol base pair $^{-1}\cdot$ K $^{-1}$ , independent of base pair type. Consequently, DNA melting temperatures vary with GC content (%GC, the percentage of duplex forming base pairs containing a G or C) according to the following relation:

$$T_m (\text{°C}) = 0.41(\%GC) + 16.6 \log[\text{Na}^+] + 81.5$$

Also included in the above equation is the dependence of the  $T_m$  on the sodium ion concentration. This is valid up to concentrations of 0.2 M and is further discussed below. For RNA duplexes, the value of the slope is higher, 0.70, with an intercept of 78 °C.

Characterizing duplex stability based solely on base pair content is useful only for estimating the melting temperatures of long nucleic acid duplexes. Even in these cases, long duplex sequences often go through multiple melting transitions due to localized regions of differing stability. The stability of a base pair can be affected by the neighboring bases due to the hydrophobic interactions between the planar, stacked bases. These stabilizing forces are sequence dependent and are typically averaged in models based solely on base pair content. Therefore, understanding the influence of base sequence on thermal stability requires a more sophisticated model, such as one that accounts for these nearest-neighbor interactions. In principle, there are 16 combinations of any two of the four different bases. However, due to the rules of base pairing, there are only ten unique double stranded combinations (for example, an AA/TT dinucleotide pair is the same as a TT/AA dinucleotide pair). Solving for their absolute contributions by comparison of melting temperatures from different sequences requires certain assumptions as the various contributions of the different stacking interactions are not linearly independent. However, various sets of values are available with a general consensus on the energetic contributions (Owcarzy et al,

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1998, SantaLucia, 1998). Explicit accounting for these interactions allows for prediction of melting temperatures to within a few degrees or less.

The nearest neighbor model is particularly useful for predicting the stability of short duplexes since the contributions of local sequence play a proportionally larger role. Short sequences, such as those typically produced using automated chemical synthesis, can show significant differences in their melting behavior when compared to long, natural DNA duplexes. For example, duplex formation requires an initiation, or nucleation, term that accounts for the energy required to form an initial base pair. For longer duplexes, this energy is inconsequential. The initiation energy is primarily entropic in nature as it relates to the bringing together of two strands. The free energy penalty for initiation is on the order of a 1-4 kcal•mol<sup>-1</sup> at 25° C and can be sequence dependent. The bimolecular nature of hybridization also leads to a concentration dependence for duplex stability. Melting temperatures for short duplexes increase with increasing strand concentration. Additionally, short duplexes tend to melt without going through intermediate, partially melted structures. These latter two features are the basis for van't Hoff thermodynamic analyses of melting curves. Considering these characteristics, assessing the stability of short duplexes requires a more refined model. A good estimation can be obtained using:

$$T^m (^{\circ}\text{C}) = 298 \frac{H^{\circ}}{H^{\circ} - G^{\circ} + RT\ln[C_T/4]} + 16.6 \log[\text{Na}^+] + 269.3$$

Where  $H^{\circ}$  and  $G^{\circ}$  account for the sum of the energetic contributions from duplex initiation, base pairing, and nearest-neighbor stacking for hybrid formation.  $C_T$  is the total strand concentration, and  $R$  is the gas constant, (1.99 cal•mol<sup>-1</sup>•K<sup>-1</sup>). The additional considerations for sequence dependent stability and concentration allow for accurate prediction of melting temperatures for short duplexes.

## **Effects of Base and Backbone Modification**

Many modifications of the standard nucleic acid bases and backbones have been studied for understanding either the role of these structures in metabolism, the forces which contribute to nucleic acid structure, or for applied uses of hybridization probes. Variations of the sugar phosphate backbone can have a significant influence on the structural and chemical properties of nucleic acids. For example, one of the distinguishing features between RNA and DNA is the use of ribose versus deoxyribose in the backbone. The additional 2'-hydroxyl group leads to both structural and reactivity differences. The hydroxyl group influences the lability of the sugar ring and adds steric hinderance but also provides an additional site for hydrogen bonding that contributes to a variety of structural conformations. The altered backbone also leads to a slightly different double helical structure and an increased duplex stability when compared to DNA.

Interest in synthetically modified backbones has resulted from their potential use as reagents for regulating gene expression. This “antisense” technology typically involves an oligonucleotide sequence that is complementary to a region of a messenger RNA or viral sequence. Binding to the target sequence leads to cleavage of the RNA by a specific enzyme or can sterically inhibit protein synthesis. By altering the backbone of antisense probes, it is possible to decrease the destabilizing phosphate-phosphate repulsions, prevent enzymatic degradation, and effect cellular delivery of the antisense probe. Many alterations have been synthesized, including

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modifications of the phosphate group, the sugar moiety, and combinations of both, or complete replacement of the backbone with an alternative polymer. Examples of the various modified backbones are presented in Figure 3. Phosphate modifications include methyl phosphonates and phosphorothioates. These phosphate modifications are generally destabilizing, however, because they reduce or eliminate the anionic charge, they allow for greater duplex stability at low counter ion concentrations. Sugar modification, such as the use of 2'-O-methylribose, form more stable hybrids than conventional nucleic acid duplexes. Other sugars, such as those based on hexopyranosyl and pento-pyranosyl structures, have been investigated to understand the etiology of nucleic acid structure (Eschenmoser, 1999). Many of these modifications actually lead to stronger base pairing properties, perhaps due to the greater rigidity of these sugars compared to that of ribose. Complete substitution of the sugar phosphate backbone with synthetic polymers have also been achieved. A peptide mimic, (PNA), where the sugar phosphate backbone is replaced by N-(2-aminoethyl)-glycine units pair effectively with DNA and RNA and increase melting temperatures by  $\sim 1.5^\circ$  per base pair for DNA:PNA and RNA:PNA duplexes (Egholm et al, 1993). Further, this uncharged backbone mimic reduces the stability dependence on salt concentration.

Several modified bases have been incorporated into nucleic acids to change hybridization specificity or stability. Naturally occurring examples are common in transfer RNA, though the function of many are unknown. Methylation of cytosine in viruses and eukaryotic DNA is common and serves an important regulatory function. In general, methylation and halogenation of the pyrimidines lead to enhanced stacking interactions and increased duplex stability. Other modifications can result from ionizing radiation and various chemical mutagens which can alter pairing affinities and structurally perturb the double helix. For example, cyclobutyl dimers between adjacent thymidine residues can result from uv irradiation and cause a large structural perturbation. On the other hand, 8-oxodeoxyguanosine, which may result from reaction with oxygen free radicals, causes minimal distortions and lowers the melting temperature of oligonucleotides only a few degrees compared to a reference G•C base pair (Plum et al, 1995). However, mispairs involving the modified guanosine are less destabilizing relative to those involving the normal base. Unfortunately, direct correlations between thermodynamic stabilities and mutation frequency or repair have not been obvious.

Other synthetic variants include several “universal” base analogs that can pair with any of the naturally occurring bases. Examples of these structures are shown in Figure 4. The base inosine, which is occasionally found in the wobble position of the tRNA anticodon loop, can pair with any of the conventional bases. The deoxyriboinosine nucleotide preferentially pairs with the DNA bases in the order cytosine > adenine > guanosine > thymine. A universal base is valuable for constructing hybridization probes when nucleotide identities are unknown or polymorphic. Additionally, a universal base can be used for lengthening short probes to increase their hybridization stability. Other universal base analogs include 3-nitropyrrole and 5-nitroindole (Bergstrom et al., 1995; Loakes and Brown, 1994). 3-Nitropyrrole is similar to *p*-nitroaniline, a known intercalator of DNA. This base substitute stacks with neighboring bases and pairs indiscriminately with the four natural bases. Melting temperatures for short oligonucleotide duplexes that contain a single 3-nitropyrrole base analog are lower than those containing only A-T and G-C base pairs. However comparison of duplex melting temperatures containing one 3-nitropyrrole base opposite one of the four canonical bases yields melting temperatures

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within a few degrees of each other. Duplexes containing 5-nitroindole have similar indiscriminate pairing properties and form more stable hybrids than duplexes containing 3-nitropyrrole.

### **Effects of Mispaired and Unpaired Bases**

Mispaired, or mismatched, bases occur when a base is opposed by any base other than its Watson-Crick partner. In DNA, there are ten potential mismatch types that involve the four bases. The stability of these structures is of interest due to its relevance to mutation frequency and DNA repair. Additionally, preventing the formation of mismatches is critical in diagnostic applications of hybridization probes. The occurrence of a mismatch is rare in DNA because of intrinsic DNA repair mechanisms, though these structures are prevalent in RNA. The normally single stranded RNA often folds upon itself and mispaired bases are frequent. The free energy values associated with mispaired bases are important for determining RNA folding by energy minimization techniques. As expected, these structures are destabilizing, relative to standard pairing, and depend on the type of mispair and the neighboring bases. Careful characterizations of their thermal stability have showed a sequence dependent hierarchy (Aboul-ela et al., 1985). Typically, mismatches involving guanine are the least destabilizing. Neighboring bases further influence mismatch stability, though a full characterization of the large number of variants is lacking. When using short hybridization probes, the position of a mispaired base within the sequence influences the extent of destabilization. Specifically, centrally located mismatches are more destabilizing than those located at the end of a duplex. Hence, for optimal discrimination, the center portion of a hybridization probe is used for assessing nucleotides of diagnostic relevance.

A single mismatched base pair is part of a larger class of structures termed internal loops. Internal loops occur when a duplexed region is interrupted by a region of unpaired bases. The loop can be symmetrical, containing an equal number of bases on both strands, or unsymmetrical, where the number of bases on the two strands may differ. Other types of loop structures include bulge loops, where only one strand contains additional nucleotides, and hairpin loops where a single strand folds back upon itself. Examples of these structures are summarized in Figure 5. In general, the occurrence of the various loop structures are rare in the normally duplexed genomic DNA, though these structures are probable intermediates during melting of long DNA duplexes. For RNA, loop structures are common. The various loop types are critical structural elements that dictate the higher order folding of RNA, participate in tertiary interactions, and serve as binding sites for ligands. Considering the large number of structural variants, complete characterization of the sequence and length dependent stabilities are lacking. A summary of experimental results can be found in (Mathews et al, 1999). In general, internal and bulge loops are destabilizing relative to duplexed sequences. Both the sequence and size of the loops can affect their stability with larger loops becoming more unfavorable. Loop stabilities are also dependent on the sequence of the adjacent duplex region. Hairpin loops are also destabilizing relative to duplexes, however, their formation is governed by a unimolecular rather than bimolecular process. Therefore, hairpin formation is often kinetically favored over bimolecular associations. Hairpin loops as small as two nucleotides are possible.

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## **Environmental Effects on Duplex Stability**

The solution environment strongly influences the stability of nucleic acid duplexes. The effects of many additives can be rationalized in terms of the forces that effect duplex stability. For example, duplex stability increases with increasing monovalent counter ion concentration. Double stranded nucleic acids have a higher charge density than when denatured. Therefore, the increased salt concentration stabilizes the phosphate - phosphate repulsion present in the duplex structure. Melting temperatures of long DNA molecules are observed to be linearly dependent on the logarithm of the monovalent counter ion concentration up to 0.2 M. The stability enhancement continues up to concentrations of ~1 M. Higher concentrations actually destabilize the duplex form. This destabilization is dependent on the nature of the anion. At high concentrations, the anions solvate the hydrophobic nucleic acid bases, stabilizing the single stranded conformation relative to the duplex form. Organic solvents have a similar, denaturing effect. The hydrophobic bases are normally stacked and protected from the hydrophilic environment when in the duplex form. However, the single strand conformation allows for greater interaction and stabilization by organic species, such as alcohols, formamide, urea or detergents, thus lowering melting temperatures. Extreme values in pH also decrease duplex stability. Melting temperatures are minimally affected between pH 5 and 9. However at lower pH values, the bases cytosine and adenine can become protonated. At higher pH values the bases guanine, thymine and uracil can become deprotonated. These altered forms can interfere with base pairing and their ionization state is more easily accomplished in the helical state. Other solution additives differentially interact with the DNA bases allowing for a leveling out of sequence dependent thermal stability. These additives include small tetraalkylammonium salts and betaine, an amino acid analog (Rees et al, 1993). At particular concentrations of these additives, a single melting temperature for long DNA molecules of varying GC content can be obtained.

## **High Order Structures - Triplexes and Quadruplexes**

Higher order complexes of nucleic acids can form under appropriate sequence and solution conditions. For example, three stranded, triplex, structures have been known since the late 1950's. This type of structure can occur at duplex regions consisting of polypyrimidine and polypurine strands. A third, polypyrimidine strand can pack into the major groove of this duplex region. This third strand is oriented parallel to the polypurine strand and is stabilized by Hoogsteen base pairing. Examples of T•A•T and C•G•C<sup>+</sup> base triplets are shown in Figure 6. Though first considered an anomaly, triplex structures have found potential use as a therapeutic, by inhibiting transcription, or as a sequence specific cleavage reagent (Moser and Dervan, 1987). Additionally, triplex formation is possible at repeating copolymer sequences, such as (dTdC)<sub>n</sub>•(dAdG)<sub>n</sub>, in a structure referred to as H-DNA. H-DNA can form as a result of negative supercoiling, where part of the polypurine strand is disassociated, allowing the polypyrimidine strand to form a three stranded complex with the remaining polypurine•polypyrimidine duplex. In general, triplex formation is favored by higher salt concentrations and increased sequence length. Furthermore, formation of a C•G•C<sup>+</sup> triplet requires protonation of the cytosine and is thus pH dependent. The association of the third strand is weaker than the parent duplex, with an association enthalpy on the order of ~2 kcal•mol triplet<sup>-1</sup> (Plum et al, 1992).

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Four stranded structure are also possible. Sequences containing tracts of guanine residues (three or more consecutive residues) can associate with other guanine tracts to form a quadruplex structure. Investigation of these structures are encouraged by the prevalence of such sequences in telomeric regions, gene promoter sites and recombination hot spots. The quadruplex is held together by a system of hydrogen bonds between the guanine bases as shown in Figure 6. This guanine tetrad can result from intramolecular folding or intermolecular annealing. Additionally, the orientation of the sequences involved in the pairings can exist in either the parallel or antiparallel conformations. The structure is stabilized by monovalent cations, favoring potassium ions over sodium ions. The G-tetrad structure is quite stable, relative to conventional Watson-Crick base pairs, with a favorable enthalpy of  $\sim 25$  kcal $\cdot$  mole tetrad $^{-1}$  and free energy at 25° of  $\sim 2.5$  kcal $\cdot$ mol $^{-1}$  (Lu et al, 1992, Jin et al, 1992).

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## Figure Legends

Figure 1#An example of an optical melting curve for a short DNA duplex. The absorbency at 268 nm was monitored as the temperature of the sample was raised from 2°C to 70°C. The resultant trace shows a sigmoidal transition. The midpoint of the transition is labelled and is referred to as the melting temperature or  $T_m$ .

Figure 2#The hydrogen bonded structures of an A-T and G-C base pair are shown. The aromatic bases lie in the same plane and are viewed from the perspective of looking down the axis of the double helix. Two hydrogen bonds are formed between the adenine and thymine bases while three hydrogen bonds are formed between the guanine and cytosine bases. The R-groups indicate the position of attachment to the sugar phosphate backbone.

Figure 3#Shown are examples of sugar phosphate backbones used in RNA, DNA and several structural variants that are described in the text.

Figure 4#Shown are nucleoside structures that exhibit degenerate base pairing properties. Shown from left are deoxyribose sugars attached to the base inosine, 3-nitropyrrole and 5-nitroindole.

Figure 5#Shown are examples of the various loop structures described in the text, including the eight different types of RNA mismatched base pairs, a symmetrical internal loop, a single base bulge loop, and a four base end loop.

Figure 6#The hydrogen bonded structures involved in triplex and G-quadruplex structures are shown.

## **Glossary**

**Melting**#Refers to the separation of a nucleic acid complex, such as the transition from a double stranded structure to the component single strands. Melting is also commonly referred to as denaturation, disassociation, or helix-coil transition and is the opposite of hybridization.

**Hybridization**#Refers to the formation of a nucleic acid complex, such as combination of two single strands to form a duplex. Hybridization is also commonly referred to as annealing, association, or duplex formation and is the opposite of melting.

**Melting Temperature**#The temperature at which 50% of duplex forming bases are paired under a specific set of experimental conditions. Also referred to as the  $T_m$ , the melting temperature is typically determined from the midpoint of the transition between the hybridized and melted states as monitored by the change in absorbance (or other property) as a function of temperature.

**Base Stacking Interaction**#The stabilizing force created between the planar, aromatic nucleic acid bases. This stabilizing force is important for the formation of various nucleic acid structures.

Figure 1.

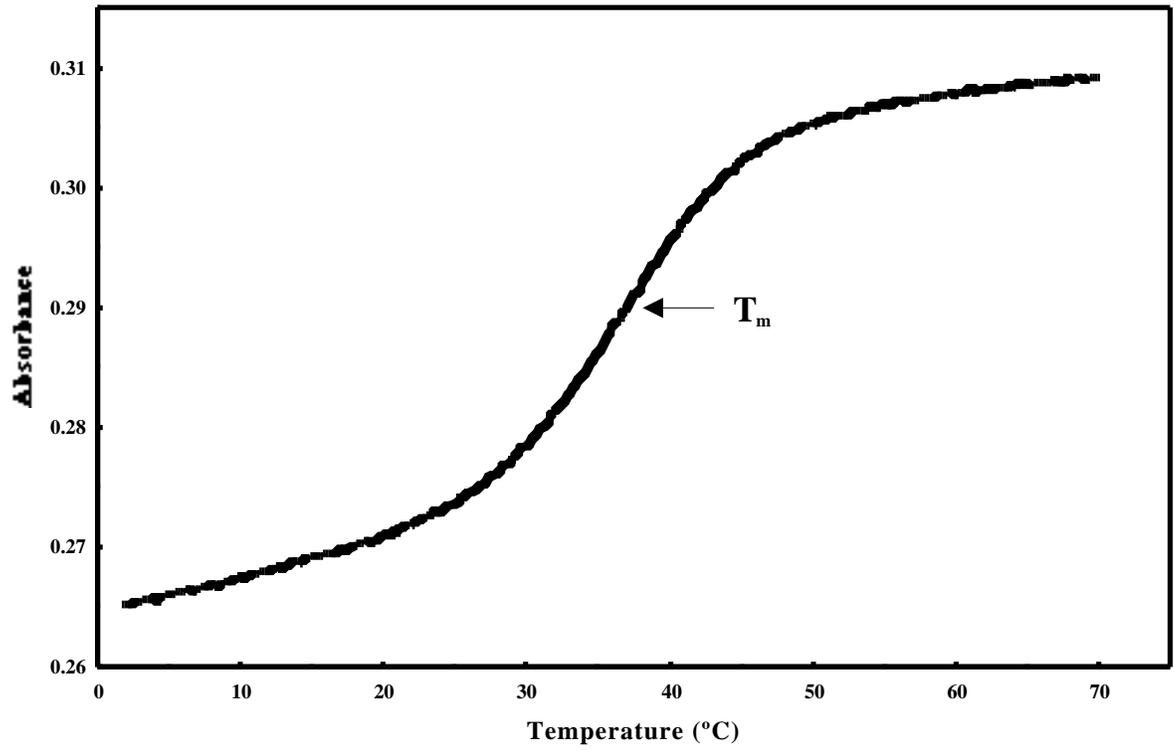


Figure 2.

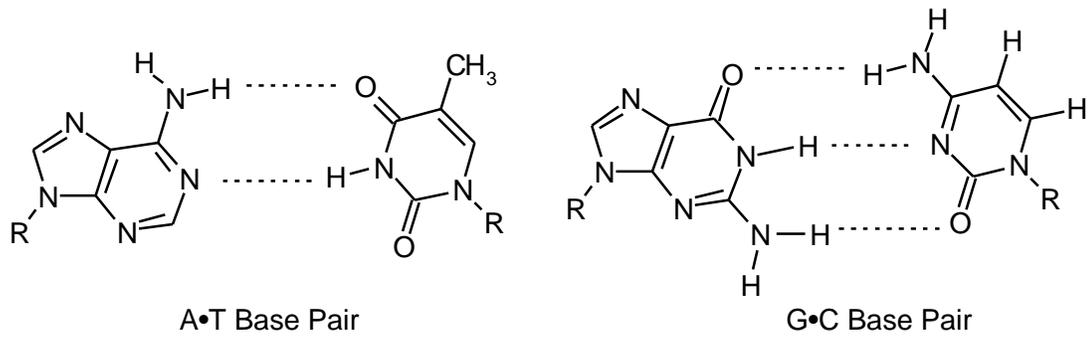


Figure 3.

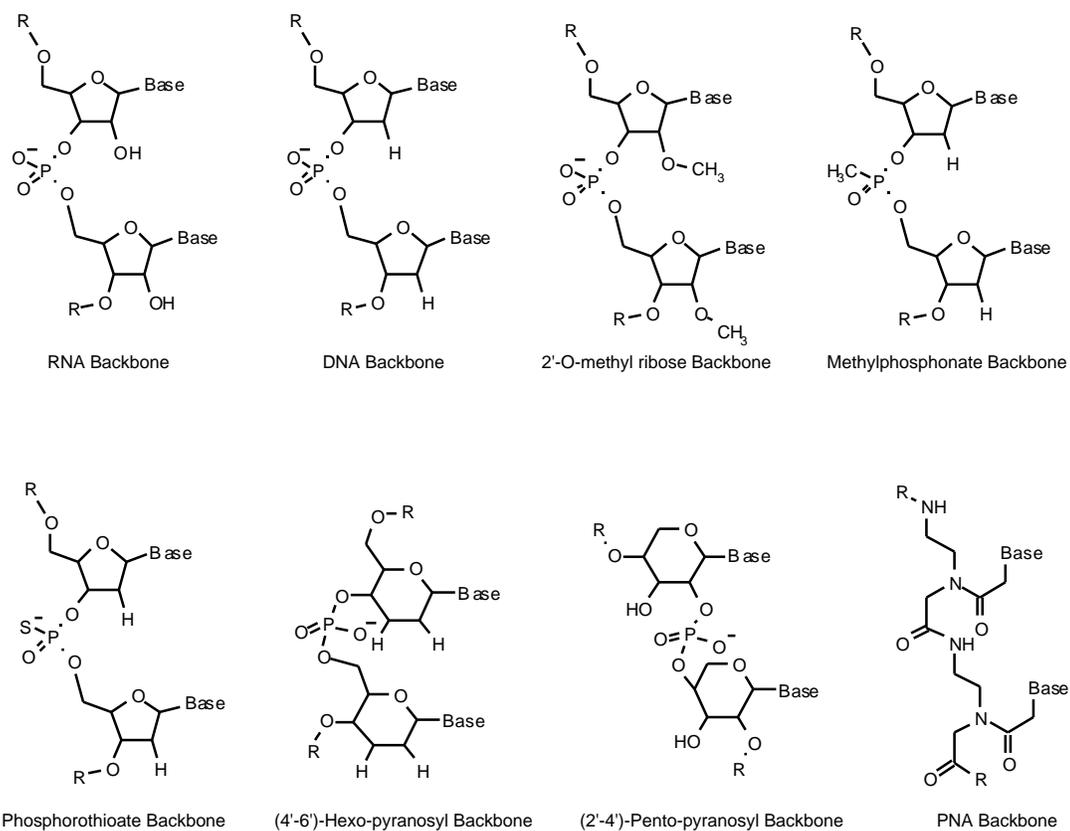


Figure 4.

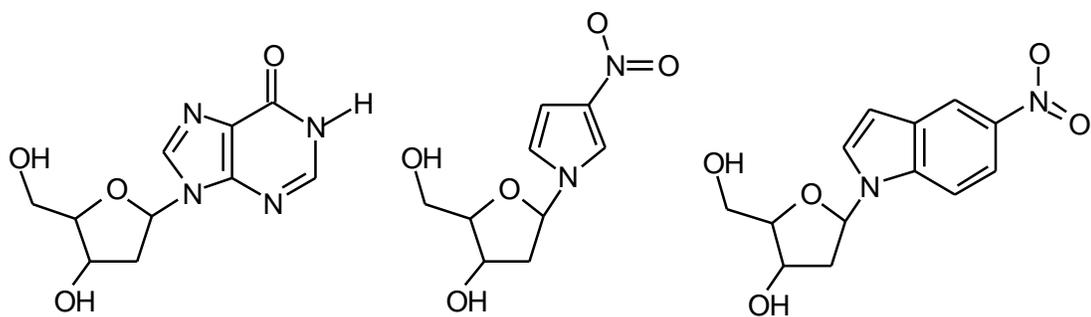


Figure 5.

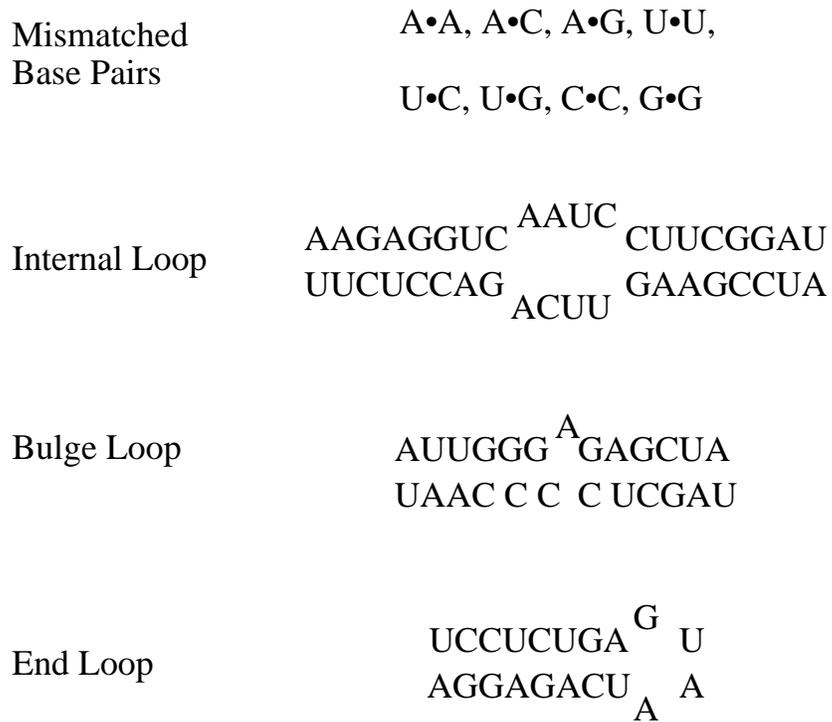


Figure 6.

