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HYDROGEN BONDING, BASE STACKING, AND STERIC EFFECTS IN DNA REPLICATION

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Key Words DNA polymerase, replication fidelity, Watson-Crick hydrogen bonding

■ **Abstract** Understanding the mechanisms by which genetic information is replicated is important both to basic knowledge of biological organisms and to many useful applications in biomedical research and biotechnology. One of the main functions of a DNA polymerase enzyme is to help DNA recognize itself with high specificity when a strand is being copied. Recent studies have shed new light on the question of what physical forces cause a polymerase enzyme to insert a nucleotide into a strand of DNA and to choose the correct nucleotide over the incorrect ones. This is discussed in the light of three main forces that govern DNA recognition: base stacking, Watson-Crick hydrogen bonding, and steric interactions. These factors are studied with natural and structurally altered DNA nucleosides.

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INTRODUCTION

The sequence-specific polymerization of DNA is central to the existence of living organisms on Earth. The chemistry of this reaction is simple: the 3′ hydroxyl group on the end of an existing primer strand forms a phosphodiester with a 5′ phosphate on a nucleotide, displacing a pyrophosphate leaving group (15, 30–32). The thermodynamic driving force is the cleavage of the weaker phosphate-phosphate bond coupled with formation of a stronger phosphodiester bond and the entropy of freeing the pyrophosphate. In principle, this reaction can occur without an enzyme, with only DNA and free nucleotides; however, it is exceedingly slow, whereas DNA polymerase enzymes can perform this at the rate of several hundred times per second. The enzyme speeds this reaction by lowering the activation barrier for the reaction by several kilocalories per mole.

The function of a DNA polymerase is to form base pairs from an existing single template strand and free nucleotides in solution, and to do so with high specificity for forming correct pairs rather than incorrect ones (15, 30–32). Thus, a polymerase helps DNA recognize itself with high specificity. To best understand how polymerases do this, it is useful first to consider how DNA alone recognizes itself, and then to understand how the enzyme influences this recognition.

We focus on what is currently understood about the forces that govern DNA-DNA recognition in the absence and presence of polymerase enzymes. First I briefly discuss each of the stabilizing and destabilizing forces with DNA alone, and then I consider what is currently known about how a polymerase utilizes each force in making DNA.

NONCOVALENT BONDING FORCES IN DNA

Hydrogen Bonding, Base Stacking, Steric Effects, and Electrostatic Effects

The structure of duplex DNA is governed by a balance of noncovalent forces in aqueous solution (27). Some forces are virtually always stabilizing; these include Watson-Crick hydrogen bonding and base stacking. Some forces are nearly always destabilizing, for example, the electrostatic repulsion of one phosphate for another along a strand and from one strand to another in the double helix. Steric effects vary in their contribution depending on what bases are matched with one another. This interplay between forces is complex and can be difficult to parse between one specific force or another. Overall, DNA double helices are strongly favored enthalpically and are disfavored nearly as much entropically (6, 53). This is commonly interpreted as a formation of many favorable bonding interactions (hydrogen bonding and base stacking) opposed by the severe entropic restrictions on the otherwise flexible backbone when the helix is formed. This is an

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oversimplification because many specific waters and metals interact strongly with the DNA helix and are thus also intimately involved in the energetics of helix formation. Nonetheless, the present discussion largely avoids issues of water and metal ion solvation for the sake of simplicity. Entropic and enthalpic effects in DNA helix formation have been discussed in detail recently (27).

NONCOVALENT BONDING IN THE POLYMERASE ACTIVE SITE

Hydrogen Bonding, Base Stacking, Steric Effects, and Electrostatic Effects

Very recently a number of important high-resolution X-ray crystal structures have been solved for DNA polymerases complexed to a primer-template duplex and with an incoming nucleotide forming the new pair (11, 12, 23, 25, 47). These reports have provided strong insights into DNA recognition in these enzymes. It is of course important to remember that the structures are snapshots of a process that involves several dynamic steps involving motions of DNA, nucleotide, and conformational changes in the protein as well. Nevertheless, this information has been very useful in the development of hypotheses for mechanistic testing of the replication process.

The DNA in a polymerase active site appears to adopt a normal right-handed helix similar to the A-form structure adopted by DNA that is in a dehydrated state. The enzyme makes contact with the end of a helix (Figure 1), binding four or five consecutive base pairs, starting with the new template base being addressed and extending approximately five base pairs downstream (corresponding to the duplex already synthesized). The active site is quite snug, closely surrounding the end of the DNA and making close contact in the major and minor grooves as well. In the minor groove are a number of hydrogen bonded contacts between amino acid side chain donors and acceptor atoms on the DNA bases in the floor of the groove (4, 11, 12, 23, 25, 47; Figure 2).

Again, the role of a polymerase is to help DNA recognize itself more selectively (see below), and so it is useful to focus on the DNA-DNA contacts in these structures, especially for the incoming nucleotide, which is the immediate substrate of the reaction. The base of the incoming nucleotide forms apparently normal hydrogen bonds with its partner, the template base (11, 12, 23, 25, 47). In addition, it is directly stacked on the terminal primer base, as it will be after the synthesis is complete. Perhaps the only unusual contact that is different between DNA alone and in the enzyme is the absence of stacking between the first template base and its immediate 5′ neighbor. In DNA alone, there are significant stacking interactions in the template single strand, whereas the polymerase bends the template DNA strand sharply, removing the next base from contact with the template base being

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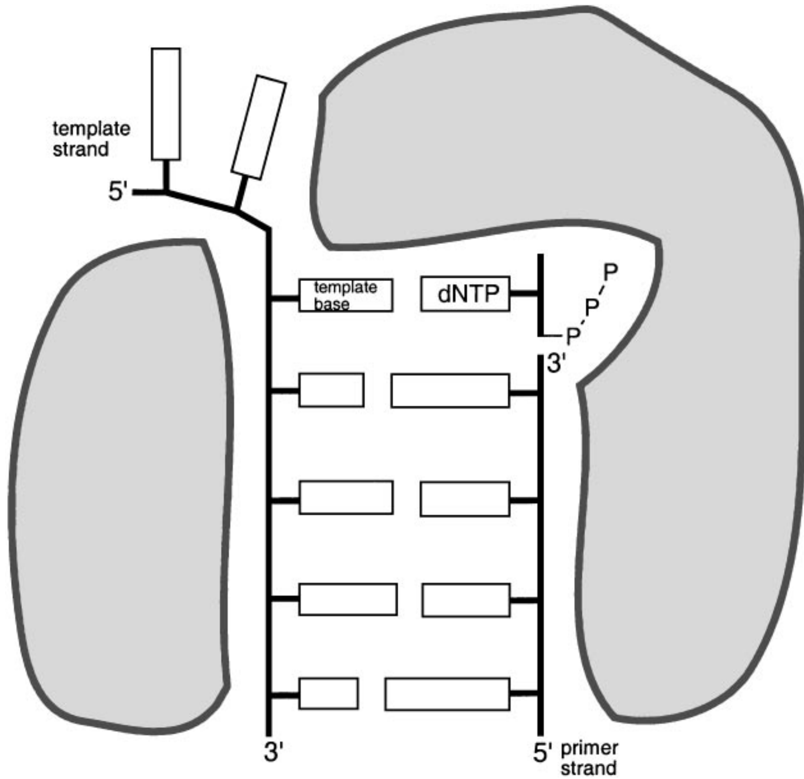

Figure 1 Schematic diagram showing how polymerases surround the end of a DNA template-primer complex, making a specifically shaped pocket for the incoming nucleotide.

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addressed (Figure 1). It is interesting that this interaction has been replaced by a stacking interaction between a tyrosine side chain directly on the template base, which may add favorable binding interactions with the DNA and also serves to limit any motion of this base.

Overall, the DNA-DNA recognition in the active site appears to be similar to what it will be after the synthesis is complete. If the enzyme does not alter the hydrogen bonding or base stacking, then how does it influence pairing selectivity so strongly? The answer may lie in steric effects (discussed below), which may be reasonable since the enzyme holds the DNA so tightly (2). Thus, the most important difference in DNA-DNA recognition may be that, in the absence of the enzyme, the ends of the DNA are quite flexible and accommodating of varied structures, whereas in the active site there may be little room for such structural alterations. These effects are discussed in more detail below.

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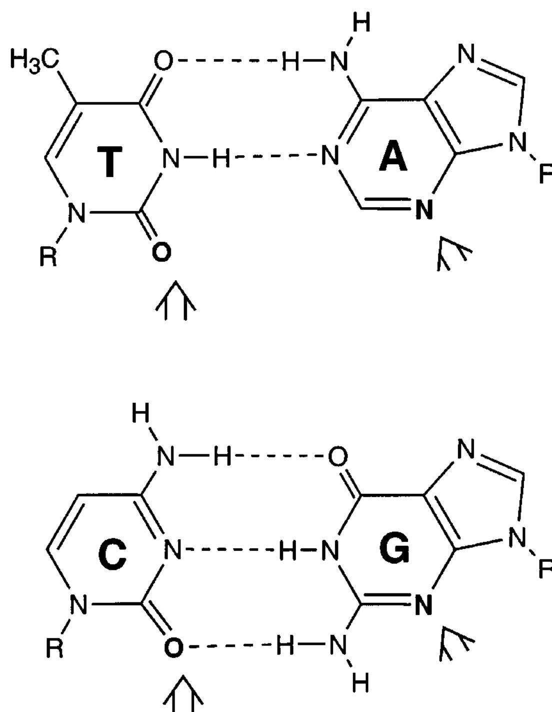

Figure 2 Structures of the two canonical base pairs, denoting the positions of minor groove H-bond acceptor atoms. Polymerases commonly form hydrogen bonds with several of these groups near the end of the DNA in the complex.

HYDROGEN BONDING IN DNA

Importance of Hydrogen Bonding in DNA Stability

To a first approximation, hydrogen bonding between two groups in water is not energetically favorable because roughly equivalent hydrogen bonds to water must be exchanged for one such new bond. Thus, in enthalpic terms, solvation effects will not favor a hydrogen bonded pairing of two nucleobases. The bases G and C must first lose several hydrogen bonds to water in order to form a triply-hydrogen bonded pair. In addition, the bases lose entropy of relative translation and rotation in order to form the complex, a destabilizing effect. However, other entropic effects favor this pairing: The entropy of the freed water molecules is likely to be favorable; moreover, the formation of the second and third H-bond in the base pair comes with little additional translational/rotational entropy penalty. This is also true as multiple pairs are formed between two strands. Thus, the hydrogen bonding in a pair does appear to be energetically favorable in the context of a larger double helix.

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How much does a Watson-Crick hydrogen bond stabilize DNA? One useful measure of this has been comparisons of doubly- or triply-H-bonded base pairs (59, 60). For example, comparison of I-C pairs to G-C pairs suggests a free energy of ca. −0.7 to −1.6 kcal/mol for this one hydrogen bond. However, the three hydrogen bonds in the pair together are worth considerably less than three times this amount because of the aforementioned cooperative effect. For example, a six-base pair duplex of G-C pairs (having 18 hydrogen bonds) may be stabilized by a free energy of −8.3 kcal/mol (20). Thus, the total stabilization per pair (including hydrogen bonding and base stacking) is −1.4 kcal, and other studies have shown that stacking accounts for probably half of this amount (see below). The above stabilization per H-bond may be considered a maximum amount with values of as low as −0.25 kcal being reasonable and perhaps more realistic. However, one important thing to take from these measurements is that the structural context strongly affects measured strengths of noncovalent interactions such as individual hydrogen bonds (54).

Another measure of the importance of hydrogen bonding to DNA stability has come from recent studies with nonpolar DNA base shape mimics. The aromatic molecule difluorotoluene has been used as a nearly perfect (but relatively nonpolar) structural mimic of thymine (20, 56, 58; Figure 3). When attached to deoxyribose, this compound (abbreviated F) forms normal DNA structure when paired with an adenine (17). Likewise, 4-methylbenzimidazole (Z) has been studied as a nonpolar mimic of adenine (although less structurally perfect in its mimicry) (18, 39). When F-Z is used to replace a T-A pair in the center of a short synthetic DNA duplex, the DNA is significantly destabilized by about 3.2 kcal/mol (50). This suggests a favorable free energy of as much as 1.6 kcal per hydrogen bond in a normal T-A pair or larger than the values suggested above. However, the importance of context is again to be stressed: When this same F-Z pair is substituted at the *end* of DNA (rather than in the middle), it is actually *more* stabilizing than

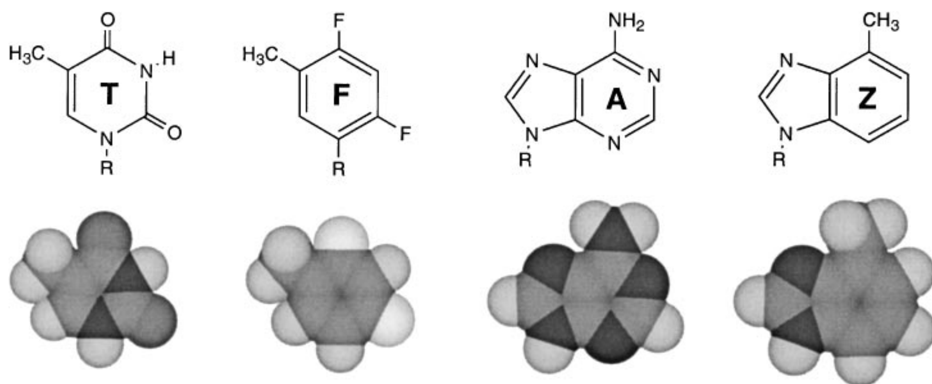

Figure 3 Structures of two natural nucleosides (A and T), alongside two nonpolar isosteres (Z and F).

a T-A pair (which suggests an unfavorable free energy of hydrogen bonding for T-A!). Two complicating factors are the differences in base stacking abilities of the natural bases and the mimics (19), and the small but significant structural distortion caused by Z because of its size, slightly larger than A (18, 39). Thus, perhaps the best measure of hydrogen bonding strength in natural DNA is the first-mentioned system, which involves smaller perturbations to the natural structure.

Watson-Crick Hydrogen Bonds and DNA Polymerases

To the first approximation, polymerases should not be strongly influenced by Watson-Crick hydrogen bonding. This is arguably true for several reasons: First, hydrogen bonding is not worth much energetically in water, particularly at the ends of DNA strands. Second, many if not all *mismatched* DNA base pairs are also linked by hydrogen bonds. Third, this bonding occurs *between* nucleobases and is internal to the DNA; thus the polymerase has no direct way to sense whether these bonds are formed or not.

Despite these facts, there has been a long-standing perception among scientists studying replication that Watson-Crick hydrogen bonds are not only important, but that they are essential to efficient and highly selective replication. This perception has been commonly reflected in textbooks (58a, 62). However, this perception has also undergone some changes among researchers in recent years as new experiments have tested this issue more directly than had been done previously (10, 13).

Because both hydrogen bonding and steric effects may have important influences on replication, perhaps the best way to test each of these effects separately is to make nucleotide analogs in which only one of the two properties is altered. For example, one might make a DNA base analog that maintains the same hydrogen bonding arrangement but has altered size or shape. This approach has not yet been examined, but another approach has: synthesis of nucleotide analogs having the same size and shape as the natural ones but lacking hydrogen bonding groups. The molecule difluorotoluene has been used as a nearly perfect (but relatively nonpolar) structural mimic of thymine (56, 58). When attached to deoxyribose, this compound (abbreviated F) forms normal DNA structure when paired with an adenine (17). Similarly, 4-methylbenzimidazole (Z) has been studied as a nonpolar mimic of adenine (7, 18, 39). These compounds have been placed into DNA to test the hydrogen bonding requirements for polymerases in adding nucleotides opposite the template, and they have been made into nucleoside triphosphate derivatives to test whether a polymerase can incorporate them into a growing primer strand.

A model for DNA polymerase activity that depends heavily on Watson-Crick hydrogen bonding would lead to some clear predictions concerning the ability of analogs such as dF and dZ to be replicated. Because they do not form such hydrogen bonds (30, 61) and are very poor at base pairing (17, 18, 58), they would be predicted to be very poor substrates for DNA polymerases. In addition, their lack of hydrogen bonds makes them virtually nonselective in their pairing abilities

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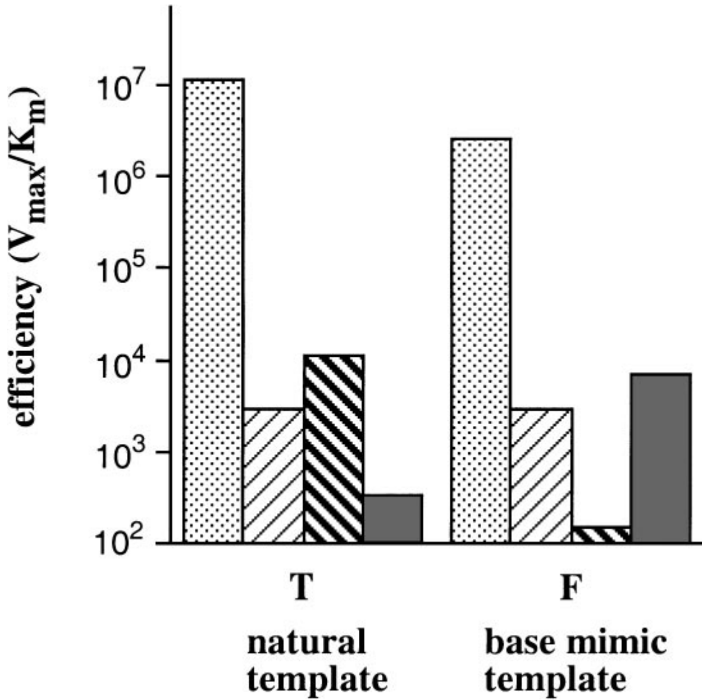

Figure 4 Steady-state efficiency for insertion of each of the four natural nucleotides opposite thymine or its nonpolar structural mimic, F (45). The enzyme is an exonuclease deficient mutant of the Klenow fragment of *E. coli* DNA polymerase I.

in DNA alone, and so it might be predicted that polymerases would process them with very low selectivity.

However, these predictions are not borne out by the evidence. It was found that dF actually serves as a highly efficient template for replication with the Kf enzyme: Adenine is inserted opposite this template base only fourfold less efficiently than opposite thymine (45). On a scale of efficiency (as V_{max}/K_m) of more than seven orders of measurable magnitude, difluorotoluene is remarkably close to a natural DNA base (Figure 4). Also important is the selectivity observed: dCTP, dGTP, and dTTP are inserted opposite dF quite poorly, at 3–5 orders of magnitude less efficient, which is essentially the same level of selectivity seen when thymine is the template base. Thus, overall the F-A base pair is processed almost the same as a T-A pair.

The reversed base pair also functions remarkably well (44). The nucleoside triphosphate analog dFTP acts as a strong polymerase substrate, allowing dF to be inserted into DNA with high efficiency. Once again, the selectivity for its insertion opposite A rather than T, C, or G is as high as is it is for the natural nucleotide

dTTP. The F-A and A-F base pairs were further tested in several sequence contexts by replicating both strands of a duplex in which one strand had all thymines replaced by difluorotoluenes (33). It appears that the only limitation of this F-A pair replication is that consecutive pairs are not well synthesized; it was proposed that this is because the primer-template duplex becomes too unstable, causing the polymerase to dissociate.

The analog dZ is also well replicated by polymerases, and the nonpolar-nonpolar pair F-Z (a wholly nonpolar analog of T-A) is processed remarkably efficiently (40). These analogs function well with a number of polymerases, including Kf, T7 DNA polymerase, HIV reverse transcriptase, and Taq polymerase. Importantly, however, not all polymerases process these nucleosides; for example, polymerase alpha and polymerase beta handle them very poorly (43). Similar results were seen for other classes of modified nucleotides (22). Thus, it is important to note that not all polymerases recognize DNA in the same way. Differences between enzymes may arise from differences in tightness of the active site and from differences in minor groove hydrogen-bonded contacts (41, 42). The reader is directed to recent reviews to find a more detailed discussion of activities with DNA polymerases (26, 28, 30).

Very recently, several new examples of efficiently replicated nonhydrogen bonding DNA base pairs have been described in the literature as well (36, 37, 46). Taken together, the results establish beyond a reasonable doubt that Watson-Crick hydrogen bonds are not needed for efficient replication of a given DNA base pair. This led to the proposal that it may be sufficient for the enzyme if two bases stack well and fit together in the DNA base pair context without serious steric clashes (28, 29).

In some cases of these new pairs, selectivity of base pair synthesis was also high; although in most of the cases the selectivity is not as high as for natural base pairs. This raises an important question about the role of Watson-Crick hydrogen bonds in selectivity/fidelity of base pair synthesis. Can selectivity be directed with steric effects alone, or do hydrogen bonds magnify the level of selectivity? Early results suggest that hydrogen bonds do increase selectivity with the natural shapes of the DNA bases. It is interesting to ask, however, whether there exist other molecular shapes that could lead to very high selectivity in the absence of hydrogen bonds. Some of the early results suggest that the answer to this may be yes (37, 46), although more work is needed. See below for more discussion of steric effects.

Interestingly, recent studies have shown that although Watson-Crick hydrogen bonds are not essential to polymerase synthesis of a given base pair, there are some selected hydrogen bonds between the polymerase and the DNA in the minor groove that are quite important for continuing the strand synthesis beyond the nonpolar base pairs (41, 42). This was established by testing a variant of dZ (called dQ) that still lacks Watson-Crick hydrogen bonding ability but now possesses a minor groove H-bond acceptor. Not only were pairs involving this analog synthesized efficiently, but DNA synthesis beyond that pair was also efficient.

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BASE STACKING IN DNA

What Is Base Stacking?

The noncovalent interaction termed "base stacking" is actually a complex interaction that depends on several noncovalent forces. "Aromatic stacking" commonly refers both to the geometry of face-to-face juxtaposition of two aromatic molecules so that the pi-systems are in direct contact, and to the forces that favor this geometry energetically. In the large majority of all known DNA structures, the bases are in face-to-face contact. They are generally not directly aligned (to maximize surface area of contact) but are rather offset. This offset orientation may be favored by the preferred conformation of the DNA backbone, and/or it may be favored by electronic effects in the bases themselves (24). In general, the distance between two aromatic planes is ca. 3.4 Angstroms in a stacked structure; this corresponds to the rise of the DNA helix per base pair.

What forces stabilize stacking in DNA? Several noncovalent forces should be considered, and all likely play a role (19). Van der Waals dispersive forces (dipole-induced dipole and induced-dipole-induced dipole attractions) certainly stabilize the stacking orientation and may play a greater role in DNA than in proteins. This is because the DNA bases are more polarizable than most amino acid side chains and because the large flat surfaces make for very intimate contact over a large area. Second, permanent electrostatic effects of interacting dipoles also undoubtedly influence stacking stability; this favorable or unfavorable contribution will depend on the bond dipoles of the molecules in question. Finally, solvation effects are also likely to affect stacking energetics, depending on whether a DNA base (most relevantly, its flat pi-surface) is better solvated by water or by a neighboring base's pi-surface.

Electrostatic effects in DNA base stacking have received the greatest research focus. This is likely because electrostatic effects are considerably easier to model computationally than dispersive forces or solvation-driven effects. On average, it appears that permanent electrostatic effects are significant in influencing variations in stability for different base pairs and structures. This largely explains why stacking efficiency of DNA bases varies considerably depending on the neighboring base. However, on average it appears that the electrostatic effects average to a very small contribution in random DNA. Thus, while one may say that electrostatic effects do locally stabilize or destabilize a given stacking interaction, and they affect the preferred geometries, it appears that on the average the electrostatic effect is small. However, more research is needed with natural bases and nonnatural analogs to evaluate electrostatic effects of altering dipole magnitudes and orientations.

A recent study of fourteen DNA bases and nonnatural analogs measured their relative stacking free energies and attempted to correlate stacking energies with molecular features (19; Figure 5). One striking feature that emerged from the study

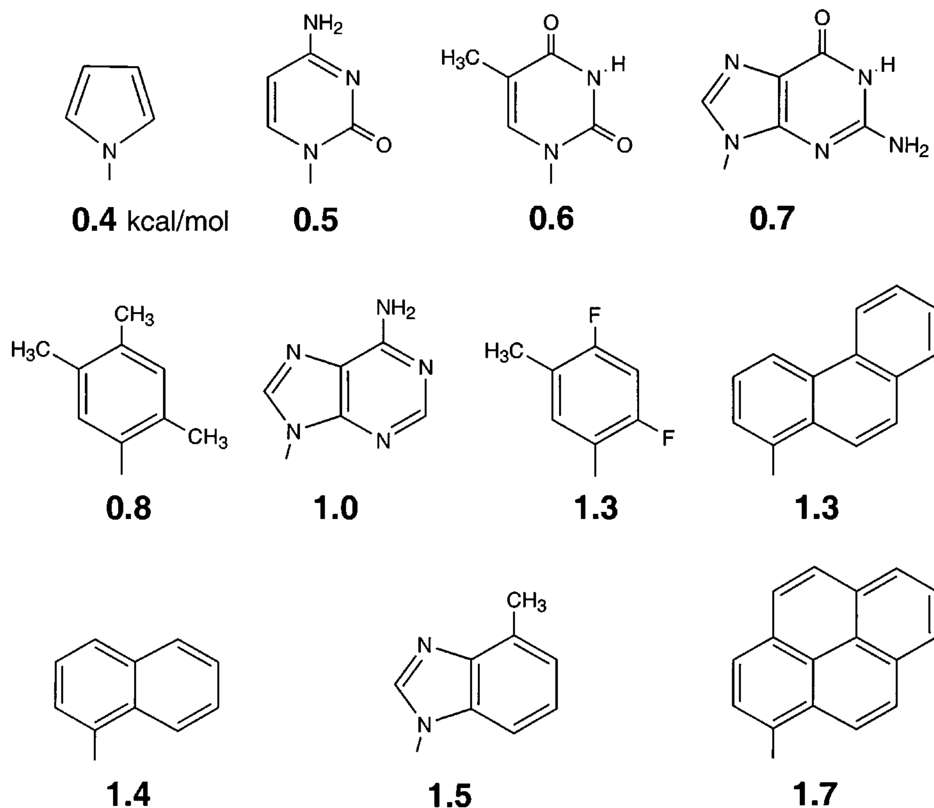

Figure 5 Stacking free energies (in kcal/mol) for a series of natural bases and nonnatural aromatic compounds, in the context of a short DNA duplex having a C-G base pair at the end (19).

is that solvent-driven forces can contribute quite strongly to stacking of aromatic groups with DNA bases. Thus, large nonpolar aromatic groups stack much more strongly than natural DNA bases do. In addition, some evidence was presented that solvent-mediated effects do stabilize stacking somewhat even for the natural DNA bases. However, the degree of this contribution relative to van der Waals effects is still uncertain. Overall, much more study is needed to gain further insight of how the three forces—dispersive, electrostatic, and solvation-driven—cooperate to stabilize stacking for the DNA bases.

Importance of Stacking to DNA Stability

Recent studies have established that the stacking of the DNA (and RNA) bases is a strong contributor to the overall stabilization of the double helix, and in fact it may be the dominant one (5, 48). Probably the best available method for measuring

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the energetics of stacking separate from hydrogen bonding is to examine single unpaired bases at the end of a duplex, where stacking is significant but no hydrogen bonded partner is present. The unpaired base can be tested on either strand, and this stacking is nearly always stabilizing to the duplex (relative to the case where the unpaired bases are lacking). For DNA the stacking of the base on the 5′ side is more favorable than on the 3′ side, presumably because the geometry of overlap is more favorable at that position. Interestingly, for RNA the reverse is true: The 3′ stacking interaction is the more favorable.

Recently a comprehensive set of data was generated for the stacking of all four natural bases on both the 5′ and 3′ sides and with all four possible neighboring bases (5). In general, the purines stack more strongly than the pyrimidines (presumably because they have larger surface area and greater polarizability). In addition, the neighboring base can have a very significant influence on stacking energetics for a given unpaired base. This may be because of different electrostatic interactions between varied pairings of the two bases directly involved in the stacking interaction and because of variable polarizabilities of those neighboring bases.

How does one derive separate numbers for stacking and hydrogen bonding for a given base pair from the above data? For a given DNA duplex, one can add an extra base pair at the end of the helix and easily derive a free energy difference for this added pair. If one assumes that this difference is an accurate measure of the total base pair's contribution to the duplex, then this favorable contribution results conceptually from three separate interactions: the stacking of the base on the 5′ side of the rest of the helix, the stacking of its partner on the 3′ side, and the hydrogen bonds between the two. Thus, if one has a total free energy value for the pair, and subtracts the free energies of the two stacking interactions (each measured separately), then the remainder should be the hydrogen bonding component. Such data were generated for RNAs quite some time ago (59) and are now available for DNA as well (5, 19, 52).

The results of these kinds of studies underscore the importance of stacking to total duplex stability. In general, the stacking of the two bases often contributes as much as, or more than, half of the free energy of the total base pair. For example, in one RNA context a terminal U-A pair adds −1.2 kcal/mol of stabilization, whereas the two stacked bases together contribute −0.8 kcal/mol without the Watson-Crick pairing component (59). In DNA, optimally placed single 5′ dangling nucleotides add up to −1.0 kcal/mol of stabilization (5, 19).

It is not yet known whether the stacking or hydrogen bonding at the end of the DNA helix differs from that in the center of the DNA. It seems possible that the greater rigidity and cooperativity in the center of a helix may influence the total pairing energies or the relative contributions of stacking and pairing in some unforeseen way. However, finding an experimental method for acquiring energetic data in the center of a strand seems to be a considerably more daunting problem than at the ends, where interactions are more isolated. In any case, it is clear that stacking is a dominant force stabilizing the helix and cannot be ignored in analyzing structure and energetics of DNA and its complexes.

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Influences of Stacking on Polymerase Activities

There is yet relatively little data in existence on the influences of base stacking on DNA polymerase activities, but it is virtually certain that stacking does play an important role. A number of lines of evidence suggest significant influences of stacking on the efficiency of DNA base pair synthesis.

There are a few cases where DNA polymerases can insert a nucleotide that is unpaired. This allows for a useful possible measure of the influence of stacking without the influence of base pairing. In templates where a base is missing (creating abasic sites), polymerases commonly add a nucleotide, albeit with low to moderate efficiency (34). Similarly, at the end of the strand a polymerase commonly adds a nucleotide beyond the end of the template, creating an unpaired base at the 3′ end of the duplex (8). In both cases, most polymerases preferentially add adenine. Studies of polymerase activities at abasic sites commonly identify adenine as the most efficient nucleotide, followed by guanine, then followed by the two pyrimidines (Figure 6). Importantly, this generally corresponds to their relative stacking abilities (5, 19). Interestingly, efficiencies and selectivities of nucleotide insertion at abasic sites significantly depend on the preceding base in the primer, on which the new nucleotide will be stacked (13). This also suggests measurable contributions of stacking on polymerase activities. Also imporant is the observation that a large nucleotide that stacks considerably more strongly than adenine can be inserted by polymerases orders of magnitude more efficiently than adenine at abasic sites (36).

Studies of the relative efficiencies of base pair synthesis for the four natural pairs usually identify the purines (A and G) as being inserted more efficiently than pyrimidines, even when the same pair is being synthesized. For example, adenine is about 10-fold more efficiently inserted opposite thymine than thymine is opposite adenine (9). This is also consistent with the relatively stronger stacking of purines over pyrimidines.

It has been noted for some time that there are "hotspots" in DNA where polymerases are more likely to make synthesis errors, leading to common mutations (3, 38). Part of what determines a hotspot is the DNA bases neighboring the mutable base pair, which again points strongly to stacking as an influence. It will be interesting to see if the recently generated stacking data for DNA will help in finding correlations between polymerase error rates and the favorable or unfavorable interactions between neighboring bases.

Of course, it should not be seen as surprising that stacking influences DNA polymerase activities. When a nucleotide binds in preparation for its insertion into the elongating primer, there are likely three main sources of favorable binding energy in the active site. The first is the binding of the triphosphate, which will probably not vary much in energy from nucleotide to nucleotide, except for possibly indirectly, where a "wrong" nucleotide is forced to adopt a poor geometry because of poor base fit. The second favorable interaction for an incoming nucleotide might be hydrogen bonding, although recent data using nonhydrogen-bonding

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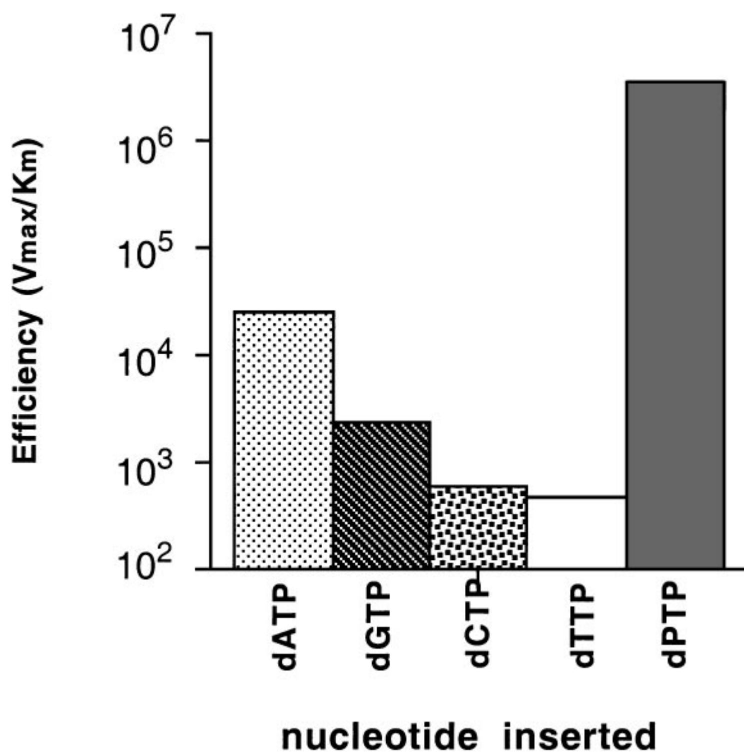

Figure 6 Relative steady-state efficiencies for insertion of the four natural nucleotides and one nonnatural nucleotide (dPTP) opposite an abasic site in a DNA template (36). The enzyme is the Klenow fragment of *E. coli* DNA polymerase I.

bases suggests (see above) that this contribution may be the least important of the three. The third favorable interaction is likely that of stacking, which we suggest may have great importance. We recently attempted to quantify this importance by synthesizing a nucleoside triphosphate analog of deoxyribose lacking a base altogether (TJ Matray, ET Kool, unpublished observations). Attempts at getting a DNA polymerase enzyme (Kf was used) to incorporate this nucleotide into a growing primer were largely unsuccessful, even under highly forcing conditions (high concentrations, long times). Kinetics studies suggest that this nucleotide, which can neither stack nor undergo hydrogen bonding, is processed at least 6 orders of magnitude less efficiently than natural nucleotides. Because we now know that hydrogen bonding can be removed and high efficiency retained, we surmise that the main problem with this nucleotide is its lack of stacking ability. Very low efficiencies have also been observed for nonnatural nucleotides having small, poorly stacking bases, whereas large, strongly stacking ones can display high efficiencies with polymerases, even at abasic sites in the template.

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STERIC EFFECTS IN DNA

Influences of Steric Effects on DNA Stability

Usually the DNA double helix is considered to be a relatively rigid structure. Certainly it is much more rigid than its component single strands. However, researchers have become increasingly aware of structures and complexes in which the DNA is strongly distorted from its native structure. Certain proteins are known to distort the helix strongly, with bending angles of 90° known (38). In addition, the DNA structural form is quite plastic, forming right-handed A-form and B-form helices, left-handed Z-form helices, parallel and antiparallel orientations, and two-, three-, four-, and even five-stranded structures.

DNA forms pairs with quite good selectivity on its own, based on the energetics of matched and mismatched pairs in the center of the helix. Singly mismatched pairs commonly cost the helix at least three to four kilocalories of free energy (26), which is probably higher than can be accounted for by that one pair itself. Thus, one surmises that the mispairing cooperatively affects neighboring DNA in an unfavorable way. Although some of this cost may arise because of differential stacking, one suspects that a majority of the energy cost comes from distortion of the pair into a nonstandard geometry, leading to unfavorable conformational interactions in neighboring pairs. It should be noted that hydrogen bonding is not likely to be a strong contributor to this cost: Many or most mismatched pairs are hydrogen bonded to one another as well (1).

How important is it that the four natural base pairs adopt virtually the same conformation, occupying almost the same space in DNA? That is, how much energy cost is associated with local structural distortions from a regular helix that might be caused by pairing of, say, a purine with a purine rather than purine with pyrimidine? The purely steric answer to this question still remains somewhat obscure, because it has been difficult to separate steric effects from stacking or hydrogen bonding effects. However, examination of a few pieces of data may shed some light on the issue.

First, examination of mismatch thermodynamic data places a limit on the free energy cost of such distortion. The worst mismatches cost ca. 4 kilocalories per mole of free energy. However, some mismatches, particularly T-G, G-G, and G-A mismatches, are less destabilizing, costing perhaps 1–2 kcal/mol and sometimes less (52). One predicts that from a steric standpoint the different mismatches should have varying degrees of stability because they adopt different conformations, and indeed they do. However, stacking effects will also vary with the base makeup of the mismatch, and thus it may be no accident that G-A and G-G mismatches are among the most stable. Overall, it still seems as if steric effects (that influence conformational effects) are indeed important. One of the most convincing reasons to believe this is the fact that mismatched DNA base pairs at the very end of a helix are not generally destabilizing to the DNA. This is consistent with the flexibility at the end of the DNA allowing the bases to adopt whatever conformation is best while

maintaining stacking. In the center of the DNA, the constraints of the backbone conformations would clearly cause some limits.

A different way to measure effects of different steric shapes in the absence of hydrogen bonding effects has been to evaluate pairing stabilities for different combinations of nonhydrogen-bonding bases having varied sizes and shapes. In general, making small changes in shapes of these bases affects the overall energetics little. For example, in evaluating the aforementioned analogs F and Z, one finds that F-F, F-Z, and Z-Z pairs differ little in energy in the center of a double helix (30). Interestingly, if one makes more dramatic changes to size by increasing it markedly, then one generally notes an increase in stability. For example, a pyrene nucleoside forms reasonably stable (mis)pairs with all four natural bases (35, 51). Models suggest, however, that in these cases the pyrene is not paired opposite a natural base, but is instead interleaved with its partner, stacked above or below it. This interleaving suggests that the pyrene has chosen to avoid bad pairwise steric congestion by adopting an alternate structure, which does present indirect evidence that pairwise steric effects may be significant, at least in systems where there is little chance of adopting a sterically reasonable structure.

Overall, the influences of steric effects in DNA (separate from proteins) are still uncertain. The picture from natural mispairs suggests that steric effects are quite significant, whereas studies with nonpolar base analogs suggest that steric effects are small. Clearly, more work needs to be done to evaluate this question.

Roles of Steric Effects in DNA Polymerization

It is interesting that the effects of sterics on polymerase activity are considerably clearer than the effects in DNA alone, without the enzyme. This is in part due to the fact that the enzymes are documented to exert strong pairing preferences in a number of cases where there is little or no preference seen in the DNA alone. Overall, the mere fact that steric effects are more clearly recognizable in DNA polymerase activity than in DNA alone suggests that the magnitude of the effects are larger in the presence of the enzyme.

Quite some time ago, it was recognized that the pairing preferences of nucleosides at the end of a DNA strand are much too small to explain the pairing fidelity that polymerase enzymes exhibit in initial base pair synthesis (49). At the time, two possible (not mutually exclusive) explanations were posited to account for this disparity. First, it was proposed that the polymerase limits the entropy at the ends of the strands by serving as a tight clamp around the helix end. The tight environment around the DNA might thus serve as a closer check on the geometry of the base pairs than occurred in DNA alone. Second, it was proposed that the enzyme might present a lower polarity environment around the DNA that might magnify the strengths of the hydrogen bonds.

Over time, the second hypothesis has become less satisfying than the first, in part because the hydrogen bonding in mismatched pairs would also be increased in strength by a lower polarity environment, just as correctly matched pairs would

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be. However, recent data suggest that the steric tightness of the active site around the DNA might indeed serve as one of the most important factors in maintaining high fidelity DNA synthesis.

How could a polymerase use steric effects to cause high pairing selectivity? Recent X-ray crystal structures of polymerases with DNA bound at the active site show the protein closely surrounding the DNA end (11, 12, 23, 25, 47, 49). If this surrounding structure is relatively rigid rather than flexible, then it presents a rigid and well-defined pocket shape in its active site. This nucleotide binding pocket has a shape defined by the enzyme wall on one side and by the template base on the other; thus, the pocket varies with the template base. A template thymine leaves just sufficient room for an adenine opposite it. We have proposed that simple steric exclusion could account for much of the selectivity seen with polymerases (28, 30). A purine would be excluded from being paired with another purine because it is simply too large. Most base mispairs could be excluded because of shapes that do not fit the overall natural base pair shape. However, at first glance it is not so easy to explain how the enzyme could exclude the small pyrimidines from being paired with one another; there clearly would be room in the active site for doing so.

Our hypothesis is that steric exclusion operates in this case as well, but by involving water as part of the steric environment (28). The natural bases carry strongly bound waters of solvation at all times. This water, it is argued, adds considerable steric size to all DNA bases in solution, thus preventing their misinsertion opposite one another by generating steric clashes in the active site. On the other hand, when a correctly matched base is inserted, it gives up these waters by exchanging them for energetically equivalent Watson-Crick bonds, becoming smaller and just the right size to fit. Thus, it is argued that, for example, thymine is not misinserted opposite another thymine because the waters on both make them too large to fit opposite one another. It should be noted that thymines can pair in a "wobble" geometry, forming two hydrogen bonds; however, this too would not be sterically allowed in the tightly defined active site.

Two lines of experimental evidence have been cited to support this reasoning. First, studies of small organic molecules in polar solvents have demonstrated that waters of solvation do indeed have real, measurable effects on structure and energetics (21). Second, recent experiments have shown that a small nonpolar mimic of thymine lacking these tightly bound waters of solvation (dF) is well inserted by polymerases opposite another dF (40). This analog of a T-T pair is processed three orders of magnitude more efficiently than is an authentic T-T pair. It is argued that in this case the dF base (i.e., difluorotoluene) is effectively smaller than thymine because it lacks the bulky waters. It should be noted that this difference between T-T and F-F pairs is seen only with a polymerase; in DNA alone, the pairs cause similar destabilizations to the DNA.

Another example of steric effects leading to high polymerase selectivities involves a large nonpolar DNA "base," pyrene. This aromatic compound occupies the surface area of a full T-A base pair. It was synthesized into a dNTP analog by attaching pyrene to deoxyribose at the C1′ position (51) and placing a 5′

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triphosphate group in the sugar. Simple steric reasoning would predict that this molecule would be poorly inserted opposite natural DNA bases or opposite another copy of itself because it is far too large. However, models of DNA suggest that it would fit well at abasic sites where the complementary base is missing altogether. Results showed that dPTP is extremely well inserted opposite abasic sites and is poorly inserted opposite itself or natural DNA bases (36; Figure 6). Again, it is relevant to note that in the absence of a polymerase, pyrene pairs quite stably with natural bases or with itself (35). Presumably this difference arises because an interleaved structure is prevented by the polymerase's tightly defined active site. One possible molecular source for prevention of such an interleaved structure may be a highly conserved tyrosine seen in all polymerases. Its aromatic ring stacks directly on top of the template base, and this very well may serve to limit its ability to move upward or downward to allow a potential partner base to slip in between.

CONCLUSIONS AND FUTURE PROSPECTS

In general, recent data with DNA alone (in the absence of enzymes) suggests that hydrogen bonds contribute strongly to the selectivity of DNA base pairing in DNA alone. The bonds also appear to contribute to pairing energetics favorably, although with only moderate magnitude. It is possible to design nonhydrogen-bonded pairs that are somewhat selective and that are at least as stable as natural base pairs. From the steric standpoint, it appears that steric effects may affect base pairing preferences somewhat, though the influence may be moderate. Finally, stacking effects are probably the major influence of base pair stability and are the major force holding the double helix together. It is interesting to note that the DNA bases stack weakly to moderately, and scientists have demonstrated many nonnatural bases that stack more strongly than the natural ones.

Importantly, it is now certain that the influences of hydrogen bonding, sterics, and stacking can be quite different in the DNA polymerase active site than with DNA alone. Measures of polymerase activities have clearly shown that Watson-Crick hydrogen bonds can be completely dispensed with, maintaining efficiencies of natural base pairs. However, it does appear that these hydrogen bonds may contribute significantly to the fidelity of synthesis. Stacking effects are now clearly documented to affect both the efficiency and fidelity of DNA synthesis in significant degrees. Finally, steric effects appear to be of primary importance in the polymerase active site, governing the efficiency and selectivity of DNA synthesis.

Because of the complexity of DNA alone in solution and the even greater complexity in the polymerase active site, there is a strong need for more studies of these factors. More research is needed to give a clearer picture of the physical phenomenon of stacking and how varied stacking affects DNA replication activities. In addition, more studies of DNA bases and analogs having widely varied shapes will provide useful new evidence about the steric exclusion model of DNA synthesis, allowing an evaluation of how much steric effects alone, even in the

absence of hydrogen bonding, can lead to high selectivity. Finally, many more DNA polymerases and other classes of polymerases (including RNA polymerases and reverse transcriptases) need to be studied in detail in order to evaluate the generality of the conclusions made in the early studies.

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