Dissociation kinetics of 19 base paired oligonucleotide-DNA duplexes containing different single mismatched base pairs

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Received September 9, 1986; Accepted November 24, 1986

ABSTRACT

The dissociation kinetics of 19 base paired oligonucleotide-DNA duplex containing a various single mismatched base pair are studied on dried agarose gels. The kinetics of the dissociation are first order under our experimental conditions. The incorporation of a single mismatched base pair destabilizes the DNA duplexes to some extent, the amount depending on the nature of the mismatched base pair. G-T and G-A mismatches slightly destabilize a duplex, while A-A, T-T, C-T and C-A mismatches significantly destabilize it. The activation energy for the overall dissociation processes for these oligonucleotide-DNA duplexes containing 19 base pairs is $52 \pm 2$ Kcalmol$^{-1}$ as determined from the slope of Arrhenius plot.

INTRODUCTION

The existence of a single mismatched base pair in an oligonucleotide-DNA duplex significantly reduces its thermal stability, when compared with the corresponding perfectly paired duplex (1,2). This effect on thermal stability allows for the formation of the perfectly paired DNA duplexes under conditions where DNA duplexes with a single mismatched base pair do not form. This requirement for perfect base pairing is the basis for methods using oligonucleotide hybridization for the identification of cloned DNA sequences (3) and for the detection of point mutations in genomic DNA (4).

Although this methodology has become an essential technique in molecular genetics (5-9), the effect of different mismatched base pairs on the stability of the DNA duplexes, is poorly understood. Without quantitative information about the effect of a single mismatched base pair, the "stringent conditions" where the perfectly matched DNA duplex will form and the DNA duplex with a single mismatched base pair will not, must be empirically determined. Experimentally, the mismatched duplex is discriminated from the perfectly matched one by two steps:

1) the hybridization efficiency of the mismatched oligonucleotide is lower than that of the perfectly matched duplex,
the dissociation of the mismatched duplex is higher than that of the perfectly matched duplex. Thus, the combination of these two factors results in the discrimination of the perfectly matched duplex from the mismatched duplex.

We have already reported the effect of hybridization temperature on the efficiency of duplex formation (1, 2, 4). In this paper, we describe the effect of different single mismatched base pairs on the rate of dissociation of oligonucleotide-DNA duplexes from a solid support. We have used hybridization of $^{32}$P-labeled synthetic oligonucleotides to the denatured human $\alpha$-globin gene bound to dried agarose gels and studied the dissociation of the labeled oligonucleotides from the supports.

**MATERIALS AND METHODS**

**Synthesis of oligonucleotides**

Four nonadecanucleotides, Probe A, C, G and T, (Fig. 1) were synthesized by the solid phase phosphotriester method using a Systec 1450 Automatic DNA Synthesizer.

**Labeling of synthetic oligonucleotides**

Synthetic oligonucleotides (23 ng, 3 pmole) were $^{32}$P-labeled using [$\gamma$-$^{32}$P] adenosine-5'-triphosphate (10 pmol, 7000 Ci/mmol) and T$_4$-polynucleotide kinase (10 units Boehringer-Mannheim) at 37°C for 30 minutes as described (10). Specific activities of the probes are approximately $1 \times 10^9$ cpm/µg.

**Preparation of DNA**

A pBR322 plasmid containing the entire normal $\alpha$-globin gene ($\alpha^A$) was obtained from Dr. Maniatis and a plasmid containing $\beta^S$-globin gene was prepared by oligonucleotide-directed mutagenesis as described previously (11). Recombinant plasmid possessing either $\alpha^A$ or $\beta^S$-globin gene (1 µg) was digested with a Bam HI endonuclease (2 units) at 37°C for 2 hours. After the digestion, the reaction mixture was extracted twice with phenol and chloroform. The restriction fragments were precipitated by ethanol as usual.

**Agarose Gel Electrophoresis and Denaturation of DNA**

Agarose gels (0.2 x 8x13 cm) were used to separate the restriction fragments. Electrophoresis was done at 200 volts for 2 hours. The following amounts of digested DNA were applied to each well: 1 µg for the mismatch experiments; 0.5 µg for the perfect match experiments. After
electrophoresis, gels were stained with ethidium bromide solution (0.5 μg/ml) for 30 minutes and photographed.

The DNA was denatured by soaking the gel in 0.5 N NaOH, 0.5 M NaCl for 30 minutes. The gel was then neutralized in 0.5 M Tris·HCl, 1.5 M NaCl, pH 8.0 for 30 minutes, washed with H2O for 30 minutes, dried on a gel drier at 80°C for 1 hour and used for hybridization.

Prehybridization

After fixing the DNA to the solid supports as described above, the solid supports were incubated in a mixture of 6xSSC (6xSSC = 0.90 M NaCl, 0.09 M sodium citrate, pH 7.0), 5 x Denhardt's (5 x Denhardt's = 0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.1% sodium dodecylsulfate (SDS) and sonicated yeast total RNA (100 μg/ml) at 60°C for 2 hours.

Hybridization

Hybridization of the fixed DNA with the labeled synthetic probes (2x 10^6 cpm/ml) were performed at room temperature for 2 hours in the same buffer used for prehybridization. The solid supports were extensively washed once with 6xSSC pH 7.0 at 30°C for 15 minutes and then at 0°C for 5 minutes and autoradiographed at room temperature for 30 minutes. Under these conditions, the extent of hybridization for the mismatched duplexes based on the counts of 32P is roughly 2-3 times less than those for the perfectly matched one, approximately 10,000 counts per 0.5 μg of the globin gene for the perfect match and 10,000 counts per 1 μg for the mismatch. The area (0.3 x 0.5 cm) at which probes hybridized was cut out and the radioactivity was measured with a scintillation counter (Beckman LS7000).

Dissociation of oligonucleotide-DNA duplexes

The dissociation of the duplexes were measured by incubating the supports in 150 ml of 6xSSC at 40°, 50° and 60°C for a given time. After a certain time of the incubation, the supports were transferred into precooled 6xSSC (4°C, 20 ml) to quench the dissociation of the probes, and rinsed thoroughly for 5 minutes to eliminate all of the dissociated probes remaining in the matrix of the supports. After the supports were dried, the radioactivity remaining on the supports was measured with a scintillation counter. In order to avoid counting non-specific binding of probes to the supports, the radioactivity remaining on an equivalent area of support containing no globin DNA was subtracted from the total counts obtained for each time point. The fraction of the duplex remaining at a time, t, Fr(t),
was calculated as the the radioactivity remaining in the supports after the thermal dissociation divided by the initial radioactivity. The fraction of the duplex released at a time, t, Fd(t), was indirectly determined from the fraction of the duplex remaining, Fd(t)=1-Fr(t). Direct measurement of Fd(t) was not practical in these experiments, because a large volume of the incubation buffer 6xSSC 150 ml) was employed. After the measurement of dissociation kinetics, the solid supports were subjected to hybridization again, and almost equal radioactivities within ±10% were counted so that there was no loss of β-globin gene bound to the solid supports, and no reannealing of denatured βA or βS gene during the course of the experiment.

RESULTS

Restriction fragments of recombinant plasmid pBR322 containing the normal human βA-globin gene and the sickle cell βS-globin gene, were denatured and fixed on agarose gels. Four synthetic oligonucleotides (Probe A,C,G and T) were hybridized to the βA and βS DNA forming eight oligonucleotide-DNA duplexes: two perfectly matched and six single mismatched base pairs (Fig. 1). All the mismatched base pairs thus exist in the same position of oligonucleotide-DNA duplexes. After the formation of oligonucleotide-DNA duplexes, the dissociation kinetics were measured at 40, 50, and 60°C. Dissociation of an oligonucleotide from the complementary DNA duplexes fixed on a solid support may involve several steps as described in Figure 2. The first step would be the fast equilibrium in which base pairs of an oligonucleotide-DNA duplex rapidly open and close with a rate constant

![Diagram](image)

**Figure 1.** A representation of the duplexes containing various single base mismatches from denatured βA-globin, (A) and βS-globin DNA, (B), with the probes (A,G,C and T).
$k_1, k_{-1}, k_2, k_{-2}, \ldots, k_n$ and $k_{-n}$ (preequilibrium). The next step would be the complete dissociation of the probe from the complementary DNA sequence with a rate constant $k_s$ (strand separation). The third step is the diffusion of the melted probe from inside the matrix to the outside aqueous solution.

Since the solid supports were thoroughly washed with a cold buffer (6xSSC at 4°C) prior to radioactivity measurements, all of the denatured oligonucleotides remaining in the matrix of the supports should be eluted. The volume of the buffer (6xSSC) used was large (150 ml) and the concentration of the released oligonucleotide was extremely low (<10^{-12} M) assuring that reassociation of the oligonucleotide released was negligible. Therefore, the overall rate of dissociation can be expressed in terms of the rate of disappearance of the DNA duplexes from the solid support,

$$v = -d[X]/dt = k [X]$$

where $k$ = overall dissociation rate constant, $[X]$ = concentration of double stranded DNA

Integrating the first order rate expression yields:

$$F_r(t) = \frac{[X]_t}{[X]_0} = \exp(-kt)$$

**Figure 2.** Diagram of the overall dissociation process of oligonucleotide-DNA duplexes. The circle represents the matrix of the solid supports. The rate constants of the preequilibria are $k_1, k_{-1}, k_2, k_{-2}, \ldots, k_n, k_{-n}$. The rate constant of the strand separation is $k_s$ and the diffusion rate constant is $k_d$, at which the denatured oligonucleotides (probes) are released from the matrix in the solid supports to the outside solution.
Figure 3. First order plot of the dissociation of the oligonucleotide-DNA duplexes bound to dried agarose gel. Each data point is the average of two experiments. Data points were taken at selected time intervals at 50°C.

○ represents A-T (perfect) base pairing; □ for G-T and △ for A-A mismatched pairs. The horizontal dotted line marks the halfway point in each DNA duplex, where $F_d(t) = 0.5$, and the corresponding times are the half-lives, $t_{1/2}$. Similar plots can be made for the other mismatched pairs.

where $F_r(t)$ = fraction of the duplex remaining in the support

$[X]_0$ = initial concentration of double stranded DNA,

$[X]_t$ = concentration of double stranded DNA at time, = $t$.

By simple Arrhenius theory, the overall rate constant may be expressed in
TABLE I.

Dissociation rate constants, $k \times 10^2$ (min) of the 19 base paired oligonucleotide-DNA duplexes containing mismatched base pairs on the dried agarose gel at 40, 50 and 60°C.

<table>
<thead>
<tr>
<th>Mismatched base pair interaction</th>
<th>A-T</th>
<th>G-T</th>
<th>A-A</th>
<th>T-T</th>
<th>G-A</th>
<th>C-T</th>
<th>C-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>T (°C) 40</td>
<td>0.5</td>
<td>0.7</td>
<td>1.0</td>
<td>1.0</td>
<td>0.8</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>50</td>
<td>4.2</td>
<td>7.0</td>
<td>13</td>
<td>11</td>
<td>8.5</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>60</td>
<td>68</td>
<td>122</td>
<td>162</td>
<td>172</td>
<td>125</td>
<td>190</td>
<td>168</td>
</tr>
</tbody>
</table>

Terms of the activation energy, $E_a$, and a preexponential factor, $A$:

$$k = A \exp\left(-\frac{E_a}{RT}\right)$$

$$\ln k = \ln A - \frac{E_a}{RT}$$

Both $E_a$ and $A$ can be found from a linear Arrhenius plot if $\ln k$ is plotted as a function of $(1/T)$.

In Figure 3 the logarithm of the fraction of the duplex remaining in the support, $\ln F_r(t)$, is plotted against time, $t$ for A-T, A-A and G-T base pairs. These plots indicate that dissociation of these DNA duplexes do indeed follow first order kinetics. The same is also true for the other duplexes (data not shown).

From this data, dissociation rate constants were obtained for the various oligonucleotide-DNA duplexes on dried agarose gel and are listed in Table 1. As expected, the rate constants for duplex dissociation increase with temperature and are larger for duplexes containing a single mismatched base pair than for that containing the perfectly matched base pair. In addition, rate constants vary with the nature of the mismatched base pair. Since all the mismatched base pairs studied in this experiment were incorporated in the same position of oligonucleotide-DNA duplexes, the magnitude of each rate constant directly reflects the relative destabilization effect of the single mismatched base pair. Consequently, the affects of mismatched base pairs can be divided into two categories:

a) **Stable mismatched base pairs** such as G-T or G-A mismatches slightly disrupt the structure of the oligonucleotide-DNA duplex so that the DNA duplex is relatively stable.

b) **Unstable mismatched base pairs** such as A-A, T-T, C-T or C-A mismatch significantly disrupt the duplex structure. As a result, the DNA duplex becomes rather labile.

As a control, dissociation kinetics of another perfectly matched base
Figure 4. Arrhenius plot of dissociation for the oligonucleotide-DNA duplexes on dried agarose gel. The activation energy, Ea, can be obtained from the slope, giving \(- 52 \pm 2\) Kcal mol\(^{-1}\) for A-T (○), G-T (□) and A-A (Δ).

pair, T-A, formed from β\(^S\)-globin DNA and probe T, was also examined at 50°C in dried agarose gel. A similar value of the rate constant (4.4 \(\times\) 10\(^{-2}\) min\(^{-1}\)) was obtained at 50°C, as compared with the other perfectly matched duplex formed between β\(^A\)-globin DNA and probe A (4.2 \(\times\) 10\(^{-2}\) min\(^{-1}\)). The total stacking energy, \(\Delta H\) of both 19 base paired oligonucleotide-DNA duplexes calculated by the nearest neighbor interaction are quite similar (\(- 156\) kcal mol\(^{-1}\)) in spite of the change of the nearest neighbor dimer unit from GAG to GTG, CTC to CAC, (13,14).

Therefore, it is reasonable that these two perfectly matched duplexes
TABLE II.

Kinetic parameters for the dissociation of the 19 base paired oligonucleotide DNA oligonucleotide duplexes on dried agarose gel and preexponential factor A, assuming constant $E_a$ equal to 52 kcal·mol$^{-1}$.

<table>
<thead>
<tr>
<th>Mismatched base pairs</th>
<th>T (°C)</th>
<th>half-life $t_1/2$ (min)</th>
<th>ln k</th>
<th>$A \times 10^{-33}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-T (perfect match)</td>
<td>40</td>
<td>139</td>
<td>-5.3</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>16.5</td>
<td>-3.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.0</td>
<td>-0.4</td>
<td></td>
</tr>
<tr>
<td>G-T (stable mismatch)</td>
<td>40</td>
<td>99</td>
<td>-5.0</td>
<td>12.6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>9.9</td>
<td>-2.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.6</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>A-A (unstable mismatch)</td>
<td>40</td>
<td>69</td>
<td>-4.6</td>
<td>18.1</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.3</td>
<td>-2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.4</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

Exhibit essentially the same dissociation rate constant. The order of the stability of the oligonucleotide-DNA duplexes containing different single mismatched base pairs in the same position is as follows:


The overall dissociation rate constants obtained in dried agarose gel at different temperatures 40°, 50° and 60°C were used in Arrhenius plots (ln k vs 1/T) to yield activation energy, $E_a$, and the preexponential factor, $A$. Figure 4 shows Arrhenius plots for the perfect match (A-T) and two mismatched base pairs, A-A and G-T. The three slopes in Figure 4 are quite similar, and can be considered to be the same, within experimental error. This slope gives activation energy, $E_a$, 52 ± 2 kcal·mol$^{-1}$. The significant difference in the Arrhenius plots is found in the Y intercept which yields preexponential factor, $A$. These values for the perfect matched duplex, and the stable and the unstable mismatched ones are listed in Table II.

DISCUSSION

We have studied the dissociation kinetics of oligonucleotide-DNA duplexes containing different mismatched base pairs. The dissociation process of all the duplexes studied in this experiment follow first order kinetics, governed by the rate constant, $k$. The magnitude of $k$ directly
TABLE III.
Activation parameters of the dissociation of the 19 base paired DNA-oligonucleotide duplexes on dried agarose gel, assuming constant Ea equal to 52 ± 2 kcal·mol⁻¹.

<table>
<thead>
<tr>
<th>Mismatched base pairs interaction</th>
<th>ΔH (kcal·mol⁻¹)</th>
<th>ΔS (cal·mol⁻¹·K⁻¹)</th>
<th>ΔG (kcal·mol⁻¹) at 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-T (perfect match)</td>
<td>51.4</td>
<td>86.6</td>
<td>25.6</td>
</tr>
<tr>
<td>G-T (stable mismatch)</td>
<td>51.4</td>
<td>87.3</td>
<td>25.4</td>
</tr>
<tr>
<td>A-A (unstable mismatch)</td>
<td>51.4</td>
<td>88.0</td>
<td>25.2</td>
</tr>
</tbody>
</table>

indicate the effect of the mismatched base pairs incorporation into the duplex.

Upon raising the temperature, the dissociation rates for all the duplexes rapidly increase to an extent depending on the nature of the incorporated mismatched base pair. However, the activation energy, Ea of the duplexes with various mismatches are very similar (52 ± 2 kcal·mol⁻¹). Therefore, the differences in the overall dissociation rate constants (among the mismatched duplexes) are entropic in origin. The activation energy (52 ± 2 kcal·mol⁻¹) for the dissociation from the dried agarose gel obtained for the A-A (unstable mismatched pair), the G-T (stable mismatched pair) and the A-T (perfect matched one) seems to be too small for the overall activation energy of the strand separation of the 19 base paired duplex, suggesting that the melting of several base pairs is the rate-limiting step of the overall dissociation process and that a pre-equilibrium step exists before strand separation. Therefore, the number of the base pairs involved in the strand separation might be 5-6 assuming that the activation energy of the individual base pair separation is about 10 Kcal·mol⁻¹ (15).

We have also evaluated activation parameters according to the Eyring equation by using the values for Ea and A obtained above:

$$ k = \frac{k_B T}{h} \exp \left( \frac{\Delta S^*}{R} \right) \exp \left( \frac{-\Delta H^*}{RT} \right) $$  

where $h$ is Planck's constant, $k_B$ is Boltzmann's constant. Comparison between equations (II) and (III) yields
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\[ A = e^{\left(\frac{kbT}{R}\right)} \exp \frac{\Delta S^\ddagger}{R} \] ---- (IV)

and

\[ \Delta H^\ddagger = E_a - RT \] ---- (V)

\[ \Delta S^\ddagger = \Delta H^\ddagger - TAS^\ddagger \] ---- (VI)

where \( \Delta H^\ddagger, \Delta S^\ddagger \) and \( \Delta G^\ddagger \) are the activation enthalpy, entropy and energy, respectively.

These data are summarized in Table III. It is noted that \( \Delta H^\ddagger \) values of the perfectly matched and the mismatched duplexes are extremely similar though the rate constant for the dissociations of the duplexes are significantly different. However, these values of activation parameters should not be emphasized too much due to the nature of the experiment employed in this study, and the rigorous study of activation parameters will be made by other experiments (16,17).

We have grouped single base mismatched base pairs into two categories, depending on the stability of oligonucleotide-DNA duplexes; stable mismatch A-G and G-T, and unstable mismatches A-A, T-T, C-T and A-C. In the two stable interactions, a guanosine base is involved as a partner. A similar high stability involving guanosine residues have been reported for the thermal stability of homopolymer and oligonucleotide duplexes containing A-G and G-G interactions (18,19). Aboul-ela et al. (20), reported on studies of mismatched bases that are incorporated between A-T base pairs. The free energy change, \( \Delta G \), of mismatched bases depends on the surrounding sequences at 25°C, but the sequence dependence is negligible at 50°C. Although the surrounding sequences are quite different, A-T (Aboul-ela) vs G-C (our studies), both thermodynamic and kinetic data for the stability of mismatched bases are fairly consistent. From the data in Table I and II, we conclude that the dissociation kinetics of oligonucleotide-DNA duplexes containing A-G or G-T single base mismatch are very similar and that the heteroduplex region does not significantly disrupt the duplexes (t1/2 = ~100 min compared to ~140 min for A-T base pair at 40°C). Among unstable mismatched base pairs, A-A, T-T, C-T and C-A, T-T and C-T destabilized the duplexes more than A-A and C-A do. This is probably due to the fact that the former mismatches include only pyrimidines which have a weak stacking effect to neighbouring bases (21) and, the latter mismatches include purines which have a higher stacking energy (22). The heteroduplex region of the unstable mismatched base pairs significantly disrupt the helical structure (t1/2 = ~70 min at 40°C). Nevertheless, the incorporation of any kind of
Figure 5. Watson-Crick model of G-C base pairing, (A), and A-T base pairing, (B). A-T has two hydrogen bonds and G-C has three. The imino protons are indicated by arrows.

A single mismatched base pair (heteroduplex) lowers the stability of the duplexes (Table 1).

The classical Watson-Crick base pair (Fig. 5) possesses the following characteristics:

i) Formation of a stable base pair between two bases requires at least two hydrogen bonds; i.e., two for A-T and three for G-C pair.

ii) One of these hydrogen bonds involved in the formation of a stable base pair interacts with the imino proton of guanosine or thymidine residues.

iii) None of four bases converts to the enol or imino tautomers in a

<table>
<thead>
<tr>
<th>Imino Proton Acceptors</th>
<th>Imino Proton Donors</th>
</tr>
</thead>
</table>

Figure 6. Diagram of the preferred base pair formations. The straight lines refer to the strong base pairing (Watson-Crick), while the dotted lines refer to weak base pairing (non-Watson-Crick).
possible keto-enol and amino-imino tautomerization of base moieties in order to form base pairing.

iv) The classical base pair is constructed from a combination of imino proton acceptor A or C with donor T or G.

v) The combination of the sterically larger purine bases A and G with the smaller pyrimidine bases C or T form sterically well balanced A-T and G-C base pairs.

A stable mismatched base pair G-T, has most of the above features (i, ii, iii and v), except that both guanosine and thymidine bases are imino proton donors, forming two $\text{NH} \cdots \text{O=C}$ hydrogen bonds (23). Another stable mismatch G-A base pair (24,25) comprises an imino proton donor G and an acceptor, A, in which, both guanosine and adenosine are purine (i, ii, iii and iv). Therefore, we expect a somewhat higher rate constant for the G-A mismatched bases duplex than the G-T mismatched duplex. Indeed, dissociation rate constants for G-A is slightly higher than that for G-T (Table I), reflecting purine-purine (G-A) base pairing having slightly more steric hindrance, compared with the purine-pyrimidine G-T pairing.

These observations lead to the hypothesis that when either or both of the two bases in a base pair have an imino proton, relatively stable hydrogen bonds can form, leading to base pairing by means of an appropriate change in the geometry of the bases. This hypothesis predicts the formation of the following base pairs T-T T-C and G-G as well as G-T and G-A. Our dissociation kinetics experiments demonstrate that T-T and T-C mismatches are quite unstable when these are incorporated into the DNA duplexes. This is probably due to the small size of the pyrimidine-pyrimidine base pairing, resulting in an excessive distortion of the helical structure. The preferred base pair combination are diagrammed in Figure 6. The solid lines correspond to the Watson-Crick base pairing (A-T and G-C), while the dotted lines correspond to weaker base pairing (G-T and G-A). This diagram predicts that the G-G pairing would be stable as G-T and G-A pairing. The formation of G-G mispairing was also predicted by thermodynamic studies of oligonucleotide duplexes (20). In contrast, A-A mismatch (purine-purine combination) does not have any imino proton so that there is no base pairing contribution to stabilize DNA duplexes.

Table IV summarizes the fraction of 19 base paired duplexes with a single mismatch remaining on the dried agarose gel, under conditions where one-half of the perfectly matched duplex dissociates. After washing at 40°C for 139 min, 50% of the perfectly matched duplex (A-T), 36% of the
Table IV
The fraction of 19 base paired oligonucleotide-DNA duplexes remaining dried agarose gel after the incubation under the condition where one perfectly matched duplex dissociates.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Washing at 40°C for 139 min</th>
<th>Washing at 50°C for 16.5 min.</th>
<th>Washing at 60°C for 1.0 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-T (perfect match)</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>G-T (stable mismatch)</td>
<td>0.36</td>
<td>0.30</td>
<td>0.29</td>
</tr>
<tr>
<td>A-A (unstable mismatch)</td>
<td>0.23</td>
<td>0.11</td>
<td>0.20</td>
</tr>
</tbody>
</table>

duplexes with stable mismatch (G-T) and 23% of the duplex with unstable mismatch (A-A) remains on the dried agarose gel, with increasing washing temperature, discrimination between the perfectly matched duplex, and mismatched duplex become more significant (see the Table).

Experimentally, a perfect matched duplex is discriminated from a mismatched duplex by two steps. The first step is in the differences in the efficiency of formation of the duplexes and the second step is in the differences in the rate of dissociation of the duplexes. Under optimal hybridization conditions (1,2), the relative hybridization efficiency of these duplexes are 1.0 (A-T), 0.5 (G-T) and 0.1 (A-A) (our unpublished results). When these duplexes are dissociated by washing the gel at 50°C for 33 min (two half-lifes of the perfect matched duplex, A-T), the ratio of these duplexes remaining in the gel would be 1.0 x (0.50)^2(A-T), 0.5 x (0.30)^2(G-T) and 0.1 x (0.11)^2(A-A) (see Table IV). If these numbers are normalized to 1.0 for AT, the values of GT and AA would be 0.18 and 0.005 respectively. The difference in the ratio would increase with increasing time of washing, thus, the discrimination of the perfectly matched duplex from any mismatched duplex is possible.

Acknowledgements
We wish to thank Dr. J.D. Opdycke and Mr. V. Hsu for their helpful discussions and Mrs. Piontkowski for the preparation of this manuscript.

This work is supported by NIH grant GM31259 and Dorothy and Earl Cohn Research Fellowship to KI.

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