The Effect of a Single-Nucleoside Gap in DNA on Cyclization Kinetics

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Introduction

Oxidizing agents and ionizing radiation damage DNA by producing gaps in one strand of a DNA duplex. Although the cell’s repair system recognizes these gaps, the details of how such damage is detected are not yet known. Thus, studying the structural and biophysical properties of gapped DNA may lead to new information on the recognition of DNA strand gaps by repair proteins. Previous experiments from our laboratory have shown that a gap generated in one strand of a DNA duplex creates a site of anisotropic bending. This finding leads one to ask whether these bends are torsionally flexible. I propose to use a cyclization assay to provide insight into the dynamics of gapped DNA. Cyclization of linear DNA can be used to measure the torsional and bending flexibility of DNA (Kahn et al., 1994). A site of localized flexibility will affect the cyclization probability (the $J$ value, $<J>$) of the DNA. These experiments are designed to measure the $J$ value, and to distinguish between three possible cases: (1) bent, torsionally flexible, (2) bent, torsionally stiff, and (3) torsionally flexible, unbent DNA. In these experiments we will expose a DNA restriction fragment to hydroxyl radical treatment to generate a library of gapped DNA molecules. The sticky ends on the restriction fragment can be ligated to form either a circle or a linear multimer. During the ligation reaction, samples are removed at various time points. A radiolabeled primer complementary to the circular DNA will be used for primer extension until a gap is reached. The DNA will be denatured prior to being run on a denaturing electrophoresis gel, with each ligation time point in a different lane. Gapped DNA that readily circularizes will produce intense bands, while less intense bands represent gapped DNA that circularizes slowly. If cyclization occurs without phase dependence, it implies that a gap is a site of torsional flexibility. Conversely, cyclization with phase dependence implies that a gap is torsionally stiff. Thus, the phase dependence of cyclization can readily be determined. The results of these experiments will contribute to our understanding of the dynamics and structure of gapped DNA.

Background

The significance of DNA as the body’s genetic map is unparalleled when compared to any other biological or chemical molecule. As a map, DNA is the blueprint for the construction of many biological molecules, thus any mutation may lead to an altered final product. One such mutation can occur when DNA polymerase encounters structurally damaged bases during replication. Repair mechanisms may function to remove the mutant base and incorporate the correct DNA base. However, when repair mechanisms fail to function the mutated base is used as a template for further replication. This results in permanent mutations that may lead to various genetic diseases (Elliott et al., 2000).

Exogenous and endogenous agents are some sources of DNA damage. Exogenous agents such as $\gamma$-radiation, UV radiation and chemical carcinogens can cause damage to DNA, one being thymine dimers (Okano et al., 2000). It is difficult for DNA polymerases to bypass thymine dimers during replication, thus halting replication and subsequent transcription reactions. Endogenous agents produced by the cell include reactive oxygen species and by-products of metabolism (Elliot et al., 2000; Cadenas &
Davies, 2000). Endogenous agents commonly cause oxidative damage to the DNA backbone and bases (Norbury & Hickson, 2000). The hydroxyl radical (\cdot OH), an oxidative species, is highly reactive and can lead to DNA damage.

The hydroxyl radical can be formed in vivo from exogenous and endogenous agents. Exogenously, water and \gamma -radiation react to form hydrogen and hydroxyl radicals (Eq. 1) (Ward, 1988).

\[
\text{H}_2\text{O} \rightarrow \cdot \text{H} + \cdot \text{OH} \quad \text{(Eq. 1)}
\]

Endogenously, the electron transport system in the mitochondria generates a superoxide anion radical by-product. Superoxide anion radicals dismutate to form hydrogen peroxide, which undergo the Fenton reaction with surrounding Fe\(^{2+}\) and Cu\(^{1+}\) to form \cdot OH (Cadenas & Davies, 2000).

\[
\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{OH} + \cdot \text{OH} \quad \text{(Eq. 2)}
\]

\[
[\text{Fe(EDTA)}]^{2-} + \text{H}_2\text{O}_2 \rightarrow [\text{Fe(EDTA)}]^{1-} + \text{OH} + \cdot \text{OH} \quad \text{(Eq. 3)}
\]

In the Fenton reaction (Eq. 2), Fe(II) reacts with hydrogen peroxide to form Fe(III), hydroxide ion, and a hydroxyl radical. The hydroxyl radical footprinting reaction (Eq. 3) developed by Tullius & Dombroski (1985) is a modification of the Fenton Reaction. In the hydroxyl radical footprinting reaction negatively charged EDTA is complexed with Fe(II) to inhibit any interactions between Fe(II) and DNA. Sodium ascorbate reduces Fe(III) back to Fe(II) for use in further hydroxyl radical reactions. Despite the very reactive nature of the hydroxyl radical, the reaction conditions can be adjusted to cleave only about 20% of the total DNA, thus ensuring at most one strand break per molecule (Tullius & Dombroski, 1985). The small size of the hydroxyl radical allows it to overcome steric clashes from the DNA backbone and bases, and react with any part of the molecule that is accessible to the solvent. Unlike the bulky structure of DNase I, the reactive and small hydroxyl radical is ideal for DNA footprinting experiments because of its ability to cleave nucleotides directly adjacent to protein-DNA contacts. Thus the hydroxyl radical reaction is an important technique for obtaining information about protein-DNA contacts.

Studies by Pogozelski et al. (1995) have shown that the hydroxyl radical generated from the hydroxyl radical footprinting reaction cleaves DNA by abstracting a hydrogen atom from the sugar moiety of DNA. The order of hydrogen abstraction corresponds to the solvent accessible surface area of the hydrogen atoms in the DNA backbone; thus, 5’ followed by 4’ hydrogen atoms are abstracted in B-DNA (Balasubramanian et al., 1998). Figure 1 illustrates the numbering scheme of hydrogen atoms in the DNA sugar moiety.

![Figure 1. Numbering scheme of hydrogen atoms in the DNA sugar moiety](image)
The removal of a hydrogen atom from the DNA backbone can produce a gap or a nick in the DNA. A nick occurs when the phosphodiester backbone is broken (Figure 2a). A gap is generated when the phosphodiester backbone is broken along with the loss of a single nucleoside (Figure 2b).

![Figure 2. The structure of (a) nicked and (b) gapped DNA](image)

DNA is not a static structure but possesses dynamic features, including curvature and flexibility. Studies have shown that DNA curvature and flexibility can be a means for DNA recognition by proteins (Dlakic & Harrington, 1995). The catabolite activator protein (CAP) is a protein which illustrates this behavior. CAP stimulates transcription of the lac promoter involved in lactose metabolism. Kahn & Crothers (1992) discovered that CAP preferentially binds its recognition sequence about 200 fold more tightly on circular molecules than on linear DNA.

Furthermore, in support of DNA bending, the crystal structure of gapped DNA with DNA Polymerase β shows an approximately 90° kink at the site of the gap (Sayawa et al., 1997). The crystal structure suggests that bending is directional. Distorted structures of bent or gapped DNA lead to anomalous electrophoretic mobility in polyacrylamide gels. It was observed by Mills et al. (1994) that constructs with 2-4 nt. gaps showed reduced electrophoretic mobility in polyacrylamide gels. In addition, hydroxyl radical gapped DNA also showed anomalous electrophoretic mobility (Guo, Dissertation 1997). Thus, the uncharacteristic gel mobility of gapped DNA and the distorted crystal structure at the site of a gap suggests that a gap could be a site of anisotropic bending.

Experiments on a 490 bp kinetoplast DNA from Leishmania tarentolae showed that it had an apparent size of 1380 bp in a 12% polyacrylamide gel (Marini et al., 1982). Sequence analysis showed that ApA steps, or A-tracts (adjacent repeating adenines), occurred ~10 bp apart. Further experiments varying the phasing between A-tracts showed that A-tracts in phase with each other resulted in additive bending. Thus, it was suggested that ApA phasings can influence the electrophoretic mobility of DNA by creating a bend in the DNA fragment. Gapped DNA, like DNA containing A-tracts, has shown similar reduced electrophoretic mobilities (Mills et al., 1994; Guo, Dissertation 1997), again indicating that a gap may induce DNA bending.
The curvature and abundance of information on A-tracts make it an excellent reference for phasing experiments of intrinsically bent DNA. The site to be tested for bending can be moved with respect to the A-tract in order to design sequences in phase or out of phase with the A-tract (Crothers et al., 1990). Two conformations can be obtained, a cis and trans conformation (Zinkel & Crothers, 1987). A test site bent in the same direction as the A-tract is in a cis conformation and has the greatest degree of bending (Figure 3a). Thus, the additive bending results in reduced electrophoretic mobility as compared to the DNA molecule containing only the A-tract. Conversely, a test site bent in the opposite direction as the A-tract is in a trans conformation and exhibits increased mobility due to an overall linear shape as compared to a fragment containing only the A-tract (Figure 3b).

![Figure 3. cis (a) and trans (b) conformations of A-tracts relative to the test sequence](image)

An experiment designed by Tullius assayed the bending at gaps created by hydroxyl radical cleavage (Guo, Dissertation 1997). A 263 bp DNA fragment contains an A-tract which creates a static bend that is used to assess the bending created by the gap. The hydroxyl radical gapped DNA is then run on a native polyacrylamide gel to determine the bending direction. If the gap is a site of bending that is phased with the A-tract, the construct will have reduced electrophoretic migration compared to non-gapped DNA. However, if the gap is out of phase with the A-tract bend, the construct will have greater electrophoretic mobility as compared to non-gapped DNA. If the gap does not create a site of bending, then gapped and non-gapped DNA run with equal mobility. The native polyacrylamide gel from (Guo, Dissertation 1997) shows a main band (band 5) and a smear above and below band #5 (Figure 4a). Thirteen slices were removed from the gel, including the main band. The DNA from each slice was electrophoresed in a separate lane on a denaturing gel (Figure 4b).

Figure 5 shows a sinusoidal pattern of electrophoretic mobility. Lanes 1-13 show a pattern repeat every 10.5 bases suggesting a phasing relationship between the A-tract bend and the gap. Lanes 1-5 show that a gap within the A-tract bent region leads to an increase in gel mobility and suggest that a gap reduces a static DNA bend. However, single nucleoside gaps along other regions of the DNA chain produce varying gel mobility. The observed phasing dependence suggests that the gap created a directional bend in the DNA (Guo, Dissertation 1997).
The nature of the bend remains unclear, and so it needs to be characterized as to its torsional and/or bending flexibility. Torsional flexibility is defined as the ability of DNA to vary its twist angle (Dlakic & Harrington, 1995). To differentiate between torsional flexibility and bending, two separate experiments can be conducted. The first experiment determines if a bend occurs and the second experiment further analyzes this particular DNA for torsional flexibility by determining which gap(s) in the backbone influence cyclization.

### Objectives

The objectives are two fold. A summary of the objectives is as follows:

- Determine if gapped DNA creates a site of bending flexibility.
- Determine if gapped DNA creates a site of torsional flexibility.

### Experimental Design

Previous experiments from our laboratory have shown that a gap generated in one strand of a DNA duplex creates a site of anisotropic bending (Guo, Dissertation 1997). This finding leads one to ask whether these bends are torsionally flexible. I propose to
use cyclization assays to provide insight into the dynamics of gapped DNA. Cyclization of linear DNA can be used to measure the torsional and bending flexibility of DNA (Kahn et al., 1994). A site of localized flexibility will affect the cyclization probability ($<J>$) of the DNA. These experiments are designed to measure $<J>$ and to distinguish between three possible cases: (1) bent, torsionally flexible, (2) bent, torsionally stiff, and (3) torsionally flexible, unbent DNA.

There are two parts to this project. Part I is meant to determine the bending flexibility. Enhanced cyclization of gapped duplex DNA is anticipated due to increased flexibility at a single nucleoside gap produced by the hydroxyl radical reaction. Part II of the project aims to determine if the bend created in Part I is torsionally flexible and if there is any phase dependence on the cyclization efficiency.

Six different size restriction fragments containing 4 $A_5\cdot T_5$ tracts with HindIII sites (AAGCTT) on the 5’ and 3’ ends will be used in the cyclization reaction. Different fragment lengths were chosen based on prior cyclization experiments (Koo et al., 1990; Kahn & Crothers, 1992; Shore et al., 1981). The persistence length, the average length of stiffness of a DNA chain, is 150 bp (Shore et al., 1981, Roychoudhury et al., 2000). Thus, fragments less than, approximately, and greater than 150 bp were chosen for this project. Furthermore, to determine the affect of phasing on curvature and flexibility, fragment lengths were chosen to be in phase (integer multiple of 10.5 bp / turn of the DNA helix) and out of phase with the A-tract (Figure 6).

### Table 1. Fragment lengths designed for cyclization

<table>
<thead>
<tr>
<th>Phase</th>
<th>Fragment Lengths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Out of phase</td>
<td>122 bp, 164 bp, 206 bp</td>
</tr>
<tr>
<td>In phase</td>
<td>126 bp, 168 bp, 210 bp</td>
</tr>
</tbody>
</table>
Part I:

To determine the bending flexibility of these fragments several experiments are conducted in parallel. The duplex DNA fragment is first radiolabeled at the 5’ end with \( ^{32}\text{P} \)-ATP. A portion of the sample undergoes ligation with T4 DNA Ligase and an aliquot of the ligated sample is treated with Exonuclease III. Meanwhile, another portion of the same sample with cohesive ends is exposed to hydroxyl radical treatment using the modified Fenton reaction (Eq. 3). The single nucleoside gaps generated by the hydroxyl radical reaction are expected to promote curvature and flexibility of the DNA helix. A portion of the gapped DNA is run on a sequencing gel to confirm that single nucleoside gaps were achieved from the hydroxyl radical reaction. The remaining hydroxyl radical treated DNA is subsequently ligated with T4 DNA Ligase. To determine the bending flexibility of the fragment and calculate \(<J>\), all of the treated samples are run on a 6% polyacrylamide gel along with a control sample of untreated DNA. For clarity, a schematic of the procedure is shown in Figure 7.

\[
<J> = \frac{K_c}{K_b} \quad \text{Measure } <J>
\]

Figure 7. Schematic of Part I experiments. a, multimers; b, cyclized DNA; c, \textit{HindIII} restriction fragments; d, cyclized gapped DNA
To determine \(<J>\) consider the equation for the cyclization reaction of a DNA restriction fragment with cohesive ends (A) into circular products (cA) by:

\[
A \xrightarrow{K_c} cA \quad (\text{Eq. 4})
\]

\[
K_c = \frac{[cA]}{[A]} \quad (\text{Eq. 5})
\]

Thus, the bimolecular reaction can be written as:

\[
2A \xrightarrow{K_b} A_2 \quad (\text{Eq. 6})
\]

\[
K_b = \frac{[A_2]}{[A]^2} \quad (\text{Eq. 7})
\]

Subtracting equation 6 from equation 4 produces a relation (Eq. 8) between the concentrations of linear and cyclic species in equilibrium.

\[
A_2 \xrightarrow{} cA + A \quad (\text{Eq. 8})
\]

\(<J>\) (Eq. 9) is the ratio of the equilibrium constant for cyclization \((K_c)\) to that for bimolecular association \((K_b)\) or the effective concentration of one end of the DNA chain around the other (Crothers et al., 1992; Levene & Crothers, 1986). Thus the ring closure probability can also be calculated from the ratio of the concentration of cyclized products to bimolecular products.

\[
\langle J \rangle = \frac{K_c}{K_b} = \frac{[cA][A]}{[A_2]} \quad (\text{Eq. 9})
\]

Part II:

Fragments that demonstrate bending flexibility will be further examined for properties of torsional flexibility. A DNA fragment with cohesive ends is treated with the hydroxyl radical (Eq. 3). The sample is then ligated under conditions for formation of monomeric circles, and aliquots are taken at various time points; however, the formation of linear multimers is unavoidable. Thus, biotinylated oligonucleotides with ends complementary to the remaining cohesive HindIII ends of linear fragments are ligated to the linear multimers. Consequently, linear multimers can be separated from cyclized DNA by running the sample through a streptavidin magnetic bead column. Cyclized DNA contained in the eluate is then used in primer extension with a \(^{32}\text{P}\)-radiolabeled primer complementary to the HindIII sequence. Primer extension occurs until a gap is reached on the cyclized DNA template. The sample is briefly denatured and run on a
sequencing gel with each time point in a different lane. A schematic of this procedure is shown in Figure 8 along with a diagram of the sequencing gel.

\[ \text{**HindIII restriction fragment**} \quad \bullet \text{OH} \quad \text{T4 DNA Ligase} \quad \circ \quad \text{or} \quad \circ \]

Ligation time

1. **Denature and run on sequencing gel**
2. **1. Add radiolabeled primer**
3. **2. Primer extension to gaps**

Figure 8. Schematic of Part II experiments

Gapped DNA that readily circularizes will produce intense bands on the sequencing gel, while less intense bands will represent gapped DNA that circularizes slowly. If cyclization occurs without phase dependence, it implies that a gap is a site of torsional flexibility. Conversely, cyclization with phase dependence implies that a gap is torsionally stiff. Thus, the phase dependence of cyclization will be determined.

This novel approach will enable me to distinguish between the three aforementioned possible cases of bending and torsional flexibility. However, as with many proposed projects, the outcome of each experiment is still very much unpredictable. From previous work on cyclization of similar length fragments (Koo et al., 1990; Kahn & Crothers, 1992; Shore et al., 1981), I believe that cyclization will occur after the introduction of a single nucleoside gap for at least one of the six different fragment lengths designed (Table 1).

**Preliminary Work**

Preliminary work has involved obtaining six DNA fragments (Table 1) with the correct sequence and HindIII restriction sites on both ends of the fragment. A pUC18 plasmid template with the correct A-tract sequence is used in PCR with primers designed to introduce HindIII restriction sites onto linear duplex PCR products. Linear PCR products are cloned into an appropriate vector and subsequently transformed into competent E. coli cells. Plasmid DNA is isolated from single colonies and sequenced, and colonies with the correct sequence are prepared for a DNA Maxi-prep. Large quantities of DNA from the Maxi-prep are further digested and run on an agarose gel. The restriction fragment is excised from the gel and purified for use in subsequent
experiments (*Part I* and *Part II*). This particular step has proved to be a difficult task because the incorporation of two *Hind*III restriction sites during PCR is extremely important for this project. After PCR, point mutations can occur throughout the cloning, transformation, and Maxi-prep steps which can make it difficult to obtain fragments with the correct sequence. Thus far I have been able to obtain the correctly phased 126 bp fragment.

**Future Work**

As stated in the Preliminary Work section, six DNA fragments with the correct sequence needs to be obtained before further experiments can be conducted. Due to the difficulty in this procedure only the phased 126 bp fragment has been obtained, thus further work is needed to acquire the remaining five fragments. Once the DNA molecules are obtained, I will begin the bending flexibility and torsional flexibility experiments (see Experimental Design section).

**Concluding Remarks**

Gaps in one strand of a DNA duplex generated from oxidizing agents and ionizing radiation are recognized by the cell’s repair system. However, the details of how such damage is detected are not yet known. Thus, information obtained from torsional and bending flexibility experiments will not only contribute to our current understanding of the dynamics and structure of gapped DNA, but also add to existing data on DNA repair.
References Cited