Electrophoretic Evidence That Single-Stranded Regions of One or More Nucleotides Dramatically Increase the Flexibility of DNA†

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ABSTRACT: The influence of single-stranded nicks and gaps on the flexibility of DNA has been investigated by subjecting to gel electrophoresis sets of molecules containing single-stranded regions of defined position and length. The DNA molecules were produced by ligating together synthetic oligomers that contained either nicks or single-stranded gaps of 1–4 nucleotides; the oligomer repeat lengths were 20, 21, 22, 23, or 26 bp, in order to produce nicks or gaps that were either in- or out-of-phase with the helix repeat of DNA. Nick-containing DNA molecules displayed nearly normal electrophoretic behavior, with maximum reductions in gel mobility (41 °C; 12% polyacrylamide gels) of approximately 10% for 230-bp molecules containing 10 nicks. In contrast, molecules containing gaps of 2–4 nucleotides demonstrated dramatic reductions in mobility, approaching one-half of the values of their full-duplex counterparts; molecules containing 1-nucleotide gaps displayed intermediate behavior. The observed (relative) mobilities of molecules containing gaps of more than 1 nucleotide were remarkably insensitive to temperature and to the presence of magnesium ions in the electrophoresis buffer. The central conclusion of the current study is that single-stranded gaps represent points of swivel-like character, whereas nicks retain much of the rigid character of double-helical DNA.

Knowledge of the structural consequences of nicks and single-stranded "gaps" in duplex DNA is essential for understanding diverse biochemical processes, including general and site-specific recombination, replication, and DNA repair. For example, in a number of viral DNAs (or viral DNA replication intermediates), there exist stable single-stranded nicks or gaps which are believed to act as initiation sites for DNA synthesis (e.g., Charneau & Clavel, 1991). Moreover, nicks and single-stranded gaps are intermediates in methyl-directed mismatch repair (Lahue et al., 1989), *uvrABC* excision repair (Caron et al., 1985; Husain et al., 1985; Kumura et al., 1985), and the SOS response (Cooper & Hunt, 1978; Hartke & Schulte-Frohlinde, 1991). Finally, undetected nicks can produce adventitious effects in many types of *in vitro* studies (Marton et al., 1991).

Single-stranded nicks and gaps are representative of a class of problems in nucleic acid structure in which the structural alteration may be either a fixed bend or an increase in flexibility. Bends are known to be induced in DNA upon binding of certain proteins to their DNA targets (Wu & Crothers, 1984; Schultz et al., 1991), through the formation of branched structures (Cooper & Hagerman, 1987, 1989; Duckett et al., 1988), or through the intrinsic curvature associated with certain sequence arrangements (e.g., "A-tracts"; for recent reviews, see Hagerman, 1990, 1992; Crothers et al., 1990). Gel electrophoresis has proven to be a powerful method for detecting and, to some extent, quantifying the degree of stable bending for these systems; however, the specific effects of points of increased flexibility on the electrophoretic mobility of DNA have not been explored in detail. This latter issue is becoming increasingly important in view of a number of recent reports in which electrophoretic behavior is being interpreted in terms of altered flexibility (Georgiadis et al., 1991; Werel et al., 1991; Kerppola & Curran, 1991).

In order to address specifically the gel behavior of DNA molecules possessing points of increased flexibility, we have constructed a series of molecules containing short, single-stranded gaps of defined sequence and location. The net effect of the gaps is a significant reduction in the electrophoretic mobilities of the gapped DNA molecules, to an extent which depends on the size of the gap.

MATERIALS AND METHODS

Chemicals and Enzymes. Acrylamide and methylene bisacrylamide were obtained from Sigma Chemical Co., as were most other reagents (acrylamide solutions were filtered through Whatman no. 1 qualitative filter paper prior to gel formation). Polynucleotide kinase and T4 DNA ligase were obtained from New England Biolabs. DNA synthesis reagents, including all phosphoramidites, were obtained from Milligen Biosearch.

Oligonucleotide Synthesis. Oligonucleotides were produced on a Biosearch 8750 automated DNA synthesizer and were cleaved from the solid-phase support by exposure to concentrated NH_4OH for 5 h at 25 °C; subsequent base deprotection was carried out in concentrated NH_4OH at 55 °C for 16 h. Oligonucleotides were then purified on preparative polyacrylamide–urea gels (20% acrylamide; 29:1 w/w monomer:bis ratio; 8 M urea). Gel slices containing DNA (visualized by brief UV shadowing) were macerated and incubated overnight in an elution buffer containing 20 mM Tris–HCl[1] (pH 8), 1 mM NaEDTA, and 50 mM NaCl. Gel particles were removed by passing the gel slurry over glass wool; the gel-isolated

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[1] Abbreviations: bp, base pairs; DE52, Whatman diethylaminoethyl cellulose; ds, double stranded; NaEDTA, sodium ethylenediaminetetraacetate; nt, nucleotide; ss, single stranded; Tris, tris(hydroxymethyl)-aminomethane.

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oligomers were further purified and concentrated by passage over DE52 (Whatman) with 3 M KOAc as the elution buffer. The oligomers were precipitated with three volumes of ethanol. DNA concentrations were determined using conversion factors of 28.5 $\mu g/A_{260}$ unit (ss oligomers) or 50.0 $\mu g/A_{260}$ unit (ds DNA).

Selected oligomers (100 μg) were phosphorylated in 200 μL of 1× LK buffer [1 mM ATP, 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM dithiothreitol] with 6 μL of polynucleotide kinase (10 units/μL) at 37 °C for 2 h. The kinase was inactivated by heating to 65 °C for 10 min followed by extraction with phenol and chloroform. Phosphorylated oligomers were then further purified by passage over a Sephadex G-25 column (Pharmacia), preequilibrated with 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA.

Annealing and Ligation of Continuous, Nicked, and Gapped Duplexes. In the current work, nicks and gaps are flanked by a 5′ OH in order to prevent their closure during the subsequent ligation reactions (Nilsson & Magnusson, 1982; Davey & Faust, 1990). Monomeric full duplexes, and nicked and gapped duplexes were prepared by mixing the appropriate single stands in 10 mM Tris and 1 mM EDTA (in 12-μL aliquots), heating to 65 °C for 2 min and air-cooling (for ~20 min) to 25 °C. The range of ss oligomer concentrations in the final annealing reactions was 0.05–0.28 $\mu g/\mu L$. In some cases, varying stoichiometries of strands were mixed, ligated, and run on polyacrylamide gels, in order to determine empirically the quantity of each component single strand necessary to produce optimal ligation. Ligation reactions did not proceed beyond the dimer stage in the absence of all three oligomers for nicked or gapped molecules. Ligation reactions (3.36–4.2 μg of annealed oligomers) were carried out in 20-μL aliquots, with 100–400 units of T4 DNA ligase in LK buffer at 25 °C for 3–15 min. Ligation reactions were terminated by the addition of NaEDTA to a final concentration of 20 mM.

Gel Electrophoresis. The ligation products were electrophoresed on nondenaturing, 6%, 9%, and 12% polyacrylamide gels (monomer:bis ratio = 37:1) at 9, 30, or 41 °C. The gel-running buffer (1× TAE) contained 40 mM Tris–HCl (pH 8.0), 20 mM NaOAc, and 1 mM EDTA, adjusted to pH 7.9 with glacial acetic acid. Gels were run at 7 V/cm. Standards used were 123- or 100-bp ladders (Bethesda Research Laboratories) as well as 23-mer and 26-mer duplex ladders. In the case of 20–22-mers, the 23-mer ladders were used, with appropriate interpolations. Gel-running temperatures ±2 °C were determined using a Sensortek implantable microthermistor embedded in test gels.

Densitometry. Gels were stained with ~1 μg/mL of ethidium bromide and photographed with Polaroid Type 665 positive/negative film. Negatives were analyzed on a Molecular Dynamics densitometer utilizing ImageQuant software. Mobilities were determined as distances from the origin of the gel to each densitometric peak and are subject to a ca. 3% measurement error. Relative mobilities of nicked or gapped molecules are reported as ratios of the observed mobilities for a given member of a ligation ladder to the mobility of the corresponding duplex species of the same length.

RESULTS

Construction of DNA Molecules Possessing Multiple Out-of-Phase Nicks or Single-Stranded Gaps. DNA molecules possessing multiple foci of stable curvature (e.g., short, homopolymeric runs of AT base pairs; A-tracts) display gel electrophoretic behavior that depends critically on the phasing of the curved regions with respect to the helix repeat: molecules with precisely phased curves may display dramatic reductions in gel mobility, whereas molecules of similar length and sequence, with curves placed every 8 or 12 bp, are often electrophoretically normal (Hagerman, 1985; Koo et al., 1986). In contrast, no dependence of mobility on phasing is expected for molecules possessing positions of increased, torsionally isotropic flexibility. Therefore, in an effort to determine the influence of single-stranded gaps on the gel behavior of otherwise double-stranded DNA molecules and whether such gaps represent points of increased flexibility, we have constructed a series of DNA molecules in which multiple nicks or gaps are at least partially out-of-phase with the helix repeat.

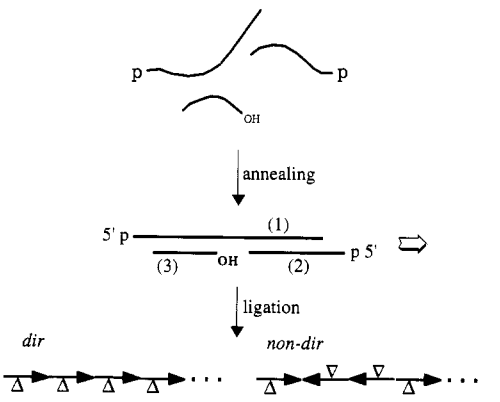

FIGURE 1: Schematic of the production of the nicked or gapped DNA molecules employed in the current study. Details of the sequence arrangements are given in Tables 2 and 3. The numerical designation of the three strands comprising the product of annealing indicates their positions relative to the nick or gap; for duplex controls, strand 2 is the complement to strand 1. The directionality (arrowhead) of the annealed species is determined by the strand containing the nick or gap (open triangle), dir and nondir refer to the sequences of the 5′ overhangs of the annealed 23-mer or 26-mer species (Table 3), with dir ends leading to directional (head–tail, ht) ligation and nondir ends leading to nondirectional (ht, hh, tt) ligation.

The current strategy for the production of DNA molecules having precisely spaced (multiple) nicks or gaps is depicted in Figure 1 and Table 1, with specific oligomer sequences and nick/gap structures provided in Tables 2 and 3, respectively. For the nicks or gaps delineated in Table 3, the strand break is terminated on both sides by hydroxyl groups, thus preventing closure of the defect during ligation. In addition, the annealed oligomers used as substrates for the ligation reactions are terminated by either non-self-complementary (5′CCGA/5′TCGG) or self-complementary (5′AGCT) overhangs (Table 2). The use of these two overhangs allows ligation of the oligomers to proceed in either a directional (dir) or nondirectional (nondir) fashion with respect to the discontinuous strand (Figure 1 and Table 1). The net effect of the use of various repeat lengths, coupled with both directional and nondirectional ligation, is the placement of nicks or gaps in various phasings with respect to the helix repeat of DNA (Figure 1 and Table 1).

DNA Molecules Possessing Multiple, Out-of-Phase, Single-Stranded Regions (Gaps) Display Dramatically Reduced Electrophoretic Mobilities on Polyacrylamide Gels. The gel electrophoretic behaviors of members of the dir series are displayed in Figure 2. It is immediately apparent that molecules possessing single-stranded regions of 2–4 nt are dramatically retarded on polyacrylamide gels, for both the 23-mer and 26-mer repeats. Identical results were obtained for 21-mers containing 2-nt gaps (data not shown). This observation implies that single-stranded regions of only a few nucleotides are points of both increased lateral and increased

Table 1: Approximate Torsional Alignment of Adjacent 3-Nucleotide Gaps

series	residual twist[a]
23-mer dir[b]	(ht)[c] 0–0.2 (turns)[d]
26-mer dir	(ht) 0.2–0.5
23-mer nondir	(ht) 0–0.2, (hh, tt) 0.5–0.7(–0.3)
26-mer nondir	(ht) 0.2–0.5, (hh) 0.3–0.6, (tt) 0.1–0.4

[a] Residual twist is expressed as a range of values, reflecting uncertainty regarding the contribution of the single-stranded region to net twist. Lower values reflect zero twist; upper values reflect normal twist (10.4–10.5 bp/turn). [b] dir refers to ligation in a directional (head–tail) fashion, due to non-self-complementary (5′CCGA/5′TCGG) overhangs; nondir refers to ligation in a nondirectional fashion, due to self-complementary (5′AGCT) overhangs. [c] ht, hh, and tt represent orientations of gap-containing oligomers (Figure 1). [d] Assuming a duplex helix twist of 10.4–10.5 bp/turn.

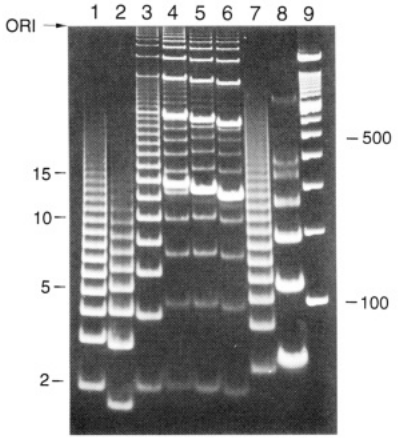

FIGURE 2: Gel electrophoretic pattern for ligated products of several nicked or gapped species in the dir series: (1) 23-mer, duplex (control); (2) 23-mer, nick; (3) 23-mer, 1-nt gap; (4) 23-mer, 2-nt gap; (5) 23-mer, 3-nt gap; (6) 23-mer, 4-nt gap; (7) 26-mer, duplex (control); (8) 26-mer, 3-nt gap; and (9) 100-bp ladder (control). The lowest band in each of lanes 1–8 represents the dimer ligation product. Numbers to the left of the photograph indicate the repeat number for the bands in lane 1; numbers to the right of the photograph indicate representative band sizes for the 100-bp ladder; ori, origin. Electrophoresis was carried out on 12% polyacrylamide at 30 °C, as described in Materials and Methods. Intense bands in the upper portions of lanes 4–6 (also detectable in lanes 3 and 8) are presumptive circular species and are being investigated further.

torsional flexibility (reduced phase dependence). Therefore, in general (e.g., for protein–DNA complexes or for structurally altered DNA), one cannot ascribe reduced electrophoretic mobility to either stable (directional) curvature or to increased flexibility, in the absence of experiments designed to test the phase sensitivity of the observed retardation. A control experiment in which the 5′ end of the 3-nt gap was phosphorylated resulted in a slight reduction (1–6%) in the mobilities of the phosphorylated series related to their nonphosphorylated counterparts (data not shown).

It is noteworthy that while DNA molecules harboring 2-, 3-, and 4-nt gaps display nearly identical, substantially reduced gel mobilities (Figure 2), molecules with 1-nt gaps migrate at rates that are intermediate between those of the ≥2-nt species and those of nicked species and normal controls. We have also investigated the phasing behavior of the nicked species by varying the repeat length from 20 to 23 bp in single base-pair increments. The curves of relative mobility vs length were all essentially identical to one another for the set of repeat lengths (Figure 3b), a result that argues against either increased flexibility or significant static bend at the nick. These observations are entirely consistent with earlier reports of both minimal distortion (Aymami et al., 1990; Snowden-Ifft & Wemmer, 1990) and absence of increased flexibility at nicks (Hays & Zimm, 1970; Shore & Baldwin, 1983). The current observation supports the notion that there is at least some base stacking across the 1-nt gap, although a more detailed structural analysis is needed to resolve this issue.

Table 2: Sequences of DNA Oligonucleotides for Electrophoretic Measurements

oligomer[a]	sequence[b]
1a	5′$_p$*CCGA*ACGTCGCATCTACGCTCGT
1b	5′$_p$*AGCT*ACGTCGCATCTACGCTCGT
1c	5′$_p$*CCGA*CACGTCGCATCTACGCTCGTCT
1d	5′$_p$*AGCT*CACGTCGCATCTACGCTCGTCC
2a	5′$_p$*TCGG*ACGAGCGTAGATGCGACGT
2b	5′$_p$*AGCT*ACGAGCGTAGATGCGACGT
2c	5′$_p$*TCGG*ACGAGCGT
2d	5′$_p$*AGCT*ACGAGCGT
2e	5′$_p$*TCGG*AGACGAGCGTAGATGCGACGTG
2f	5′$_p$*AGCT*GGACGAGCGTAGATGCGACGTG
2g	5′$_p$*TCGG*AGACGAGCGT
2h	5′$_p$*AGCT*GGACGAGCGT
3a	5′$_{HO}$AGATGCGACGT
3b	5′$_{HO}$GATGCGACGT
3c	5′$_{HO}$ATGCGACGT
3d	5′$_{HO}$TGCGACGT
3e	5′$_{HO}$GCGACGT
3f	5′$_{HO}$TGCGACGTG

[a] Numerical designation refers to the single-stranded species as specified in Figure 1. Species 2a,b,e,f refer to the complements (with 5′ overhangs) to species 1a,b,c,d, respectively. [b] 5′ overhangs are indicated in italics. For molecules of the 20-, 21-, and 22-bp series, oligomers 1a and 3a are shortened by 3, 2, or 1 nt, respectively, at the positions underlined.

One additional feature of Figure 2 should be noted, namely the presence of a series of intense bands in the upper half of lanes 4–6. We are currently investigating these bands, which we believe to comprise circular DNA molecules whose formation is facilitated by the flexible gaps. These bands are also present following ligation of DNA molecules with 1-nt gaps (Figure 2, lane 3); however, we have not observed such species with DNA molecules containing out-of-phase nicks.

The foregoing results pertain to the electrophoretic behavior of DNA molecules with nicks or gaps that have been produced through directional ligation. However, essentially identical gel behavior is displayed (Figure 3) by 3-nt gap-containing molecules (both 23-mer and 26-mer series) in which ligation is not directional. Since these latter series give rise to additional phase relationships (Figure 1 and Table 1), their similar electrophoretic behavior is further evidence of the absence of a strong directional component to the increased flexibility of small, single-stranded regions.

Temperature Dependence of the Relative Mobilities for DNA Molecules Possessing Out-of-Phase Nicks or Gaps. The gel behaviors of nicked or gapped molecules within the 23-mer dir series are displayed in Figure 4 for three gel temperatures (9, 30, and 41 °C). It is evident that the mobilities of molecules harboring gaps of at least 2 nt are not markedly influenced by the gel-running temperature within that temperature range. This observation is consistent with the characterization of single-stranded regions of 2–4 nt as flexible hinges, without substantial restriction of either torsional or lateral deflections. It should be noted that the sequences of the bridging (single-stranded) regions used in the current study (Table 3) were chosen to minimize intrastrand stacking interactions in order to examine the gel behavior of molecules with centers of flexibility. It is certainly possible that single-stranded regions comprising runs of A or G residues would display temperature-dependent, restricted motion (Eisenberg & Felsenfeld, 1967; Inners & Felsenfeld,

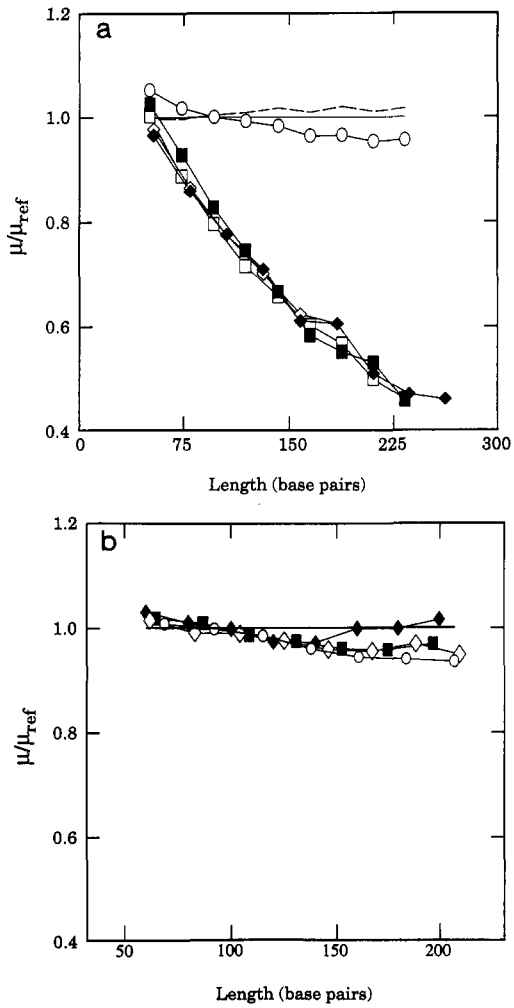

FIGURE 3: (a) Plot of the relative electrophoretic mobilities of sets of DNA molecules possessing either nicks or 3-nt gaps for three distinct periodicities of the nicks/gaps: (○) 23 bp (dir), nick; (□) 23 bp (dir), 3-nt gap; (■) 23 bp (nondir), 3-nt gap; (◇) 26 bp (dir), 3-nt gap; and (◆) 26 bp (nondir), 3-nt gap. All mobilities are plotted relative to the mobilities of the 23-bp (duplex) dir control set; dashed line, 23-bp (duplex) nondir control set. (b) Plots of 20 (◆), 21 (◇), 22 (■), and 23-mer (○) nicked repeats, using the 23-mer duplex series as a control. Electrophoresis experiments were performed as described for Figure 2. (T = 30 °C).

1970; Ackter & Felsenfeld, 1971; Stannard & Felsenfeld, 1975).

In contrast to the near temperature independence of the mobilities of the gapped molecules discussed above, molecules possessing nicks do demonstrate a small reduction in mobility with increasing temperature (Figure 4). This behavior is consistent with the partial disruption of base stacking at the nick sites, leading to increased motional freedom at the nick for the higher temperatures. The intermediate degree of retardation for molecules possessing 1-nt gaps is indicative of restricted motion within that gap. Furthermore, the slight temperature dependence of the mobilities suggests that this restriction may be steric, with residual base-stacking interactions contributing to a lesser degree. A modest reduction in the relative mobilities of the 23-mer, 3-nt gap species with increasing percent acrylamide (6%, 9%, 12%) was also noted (≤10% reduction over the range 6–12% acrylamide; data not shown). This last observation may reflect the added difficulty in maintaining directional movement for molecules possessing points of hingelike flexibility.

Magnesium Ions Have Little Effect on the Relative Electrophoretic Mobilities of Molecules Possessing Either Nicks or Gaps. The electrophoretic behavior of both nicked and gapped molecules in the presence of magnesium ions is displayed in Figure 5. Comparison of this set of curves with those in Figure 3b reveals that magnesium ions have little

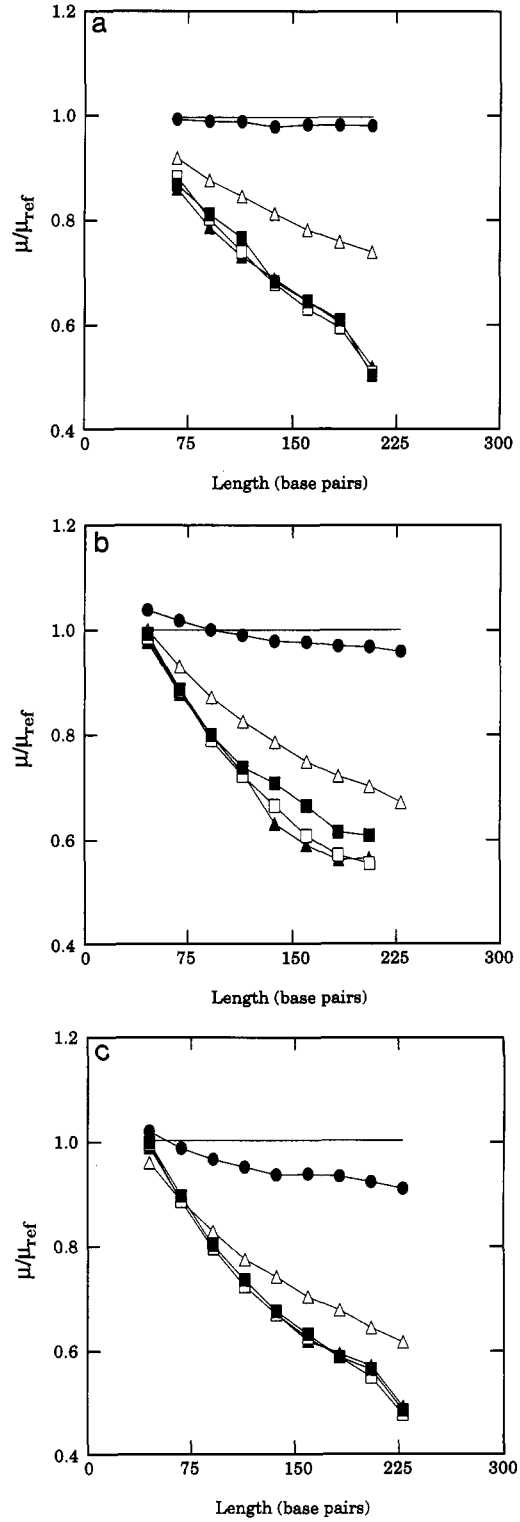

FIGURE 4: Plots of the relative electrophoretic mobilities, μ/μ_{ref}, of sets of molecules comprising multiple copies of directionally ligated (dir) 23-bp oligomers (Table 1). Each 23-mer contains either a nick (●), 1-nt single-stranded gap (△), 2-nt gap (▲), 3-nt gap (□), or 4-nt gap (■), as detailed in Table 3. The reference mobilities, μ_{ref}, are obtained for a set of DNA molecules comprising full-duplex 23-mers and whose members all display normal electrophoretic behavior: (a) 9 °C, (b) 30 °C, and (c) 41 °C. Electrophoresis experiments were performed on 12% polyacrylamide gels (see Materials and Methods).

Table 3: Duplex Oligomeric Substrates for T4 DNA Ligase Reactions

oligomer[a]	annealed products[b,c]	
1a, 2a	(5′) •••C A T C T A C••• •••G T A G A T G••• (5′)	duplex control
1a, 2c, 3a	•••C A T C T A C••• •••G T A G A_{OH} T G•••	nick
1a, 2c, 3b	•••C A T C t A C••• •••G T A G_{OH} T G•••	1-nt gap
1a, 2c, 3c	•••C A T c t A C••• •••G T A_{OH} T G•••	2-nt gap
1a, 2c, 3d	•••C A t c t A C••• •••G T_{OH} T G•••	3-nt gap
1a, 2c, 3e	•••C a t c t A C••• ••• G_{OH} T G•••	4-nt gap
	23-mer (nondir)	
1b, 2b	*e*	duplex control
1b, 2d, 3a		nick
1b, 2d, 3d		3-nt gap
	26-mer (dir)	
1c, 2e		duplex control
1c, 2g, 3f		3-nt gap
	26-mer (nondir)	
1d, 2f		duplex control
1d, 2h, 3f		3-nt gap

[a] Sequences of single-stranded oligomers are listed in Table 1. [b] The region of the helix adjacent to the nick or gap is delineated, with strand polarities as indicated for the 23-mer (dir) duplex control. 5′ hydroxyl groups are indicated to emphasize the absence of a 5′ phosphate at the nick/gap site. [c] Single-stranded nucleotidyl residues are indicated in lower case bold. [d] nondir, 5′AGCT overhangs; dir, 5′CCGA/5′TCGG overhangs. [e] Nick or gap regions not depicted are identical to those of the corresponding 23-mer (dir) species.

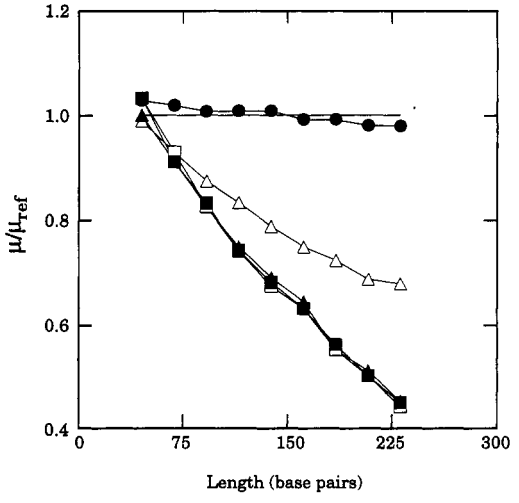

FIGURE 5: Plot of the relative electrophoretic mobilities in the presence of $MgCl_2$ of the 23-mer series described in Figure 4. Electrophoresis was performed as described in Figure 4 (Materials and Methods), except for the addition of 5 mM $MgCl_2$ to the gel-running buffer (T = 30 °C).

effect on the apparent flexibility displayed by any of the disruptions. This last observation also suggests that the reduced mobilities are not a sensitive function of differing degrees of phosphate-charge neutralization secondary to counterion valence. Moreover, the addition of K glutamate (final concentation, 40 mM) and $MgCl_2$ (final concentration, 5 mM) to the standard 1× TAE buffer (in both gels and running buffer) had no noticeable effect on the relative mobilities of the 23-mer nick or 3-nt gap species (data not shown).

Finally, it should be noted that several of the dimeric species (e.g., the 46-bp species containing two nicks) display slightly higher mobilities than those of the corresponding duplex controls. While this behavior may reflect intrinsic differences in the migration of those nicked species, it is also possible that those species have simply lost one of the the single-stranded oligomers from one end of the molecule. This latter effect would not be expected to alter substantially the mobilities of the longer molecules.

DISCUSSION

Physical Characteristics of Single-Stranded Nicks in DNA. The electrophoretic mobilities of DNA molecules possessing one nick every 20, 21, 22, or 23 bp are quite similar to those of their full-duplex counterparts (Figure 3b). This observation suggests that a nick imparts neither substantial additional flexibility nor a significant static bend to the helix, in contrast to the behavior of gaps (below) and in complete agreement with earlier work (Thomas, 1956; Schumaker et al., 1956; Hays & Zimm, 1970; Shore & Baldwin, 1983). Hays and Zimm (1970) employed sedimentation velocity and intrinsic viscosity measurements to characterize the overall flexibility of DNA molecules that contained up to five nicks per persistence length (ca. 1 nick/30 bp). They observed essentially no difference in hydrodynamic behavior between nicked and duplex DNA at temperatures up to about 40 °C, under conditions where a reduction of 20–30% in the overall persistence length would have been detected. Shore and Baldwin (1983) employed a sensitive, DNA cyclization assay to demonstrate that, at 20 °C, singly nicked DNA molecules resisted torsional release (the swiveling of one strand about the other at a nick) under nearly 2 kcal of twisting free energy. Those authors argued that preservation of base-stacking interactions at the nick prevents free torsional motion. It is, of course, well known that nicks in supercoiled DNA molecules release supercoils; however, the attendant molar free-energy release for the latter process is often tens to hundreds of kilocalories.

In at least two studies, nicks have been introduced at the centers of DNA recognition sequences whose structures are distorted by their associated proteins. As part of their investigation of the sequence requirements for binding of the bacteriophage 434 repressor to the 434 operator, Koudelka et al. (1988) introduced a nick at the center of the operator. They observed a modest (5-fold) increase in binding affinity and attributed this effect to increased flexibility at the nick site. Giraud-Panis et al. (1992) performed a similar experiment, introducing a nick at the center of the recognition sequence for binding of *Escherichia coli* cyclic AMP receptor protein (CRP). CRP is known to bend DNA (Wu & Crothers, 1984; Kotlarz et al., 1986; Dripps & Wartell, 1987; Schultz et al., 1991), and Giraud-Panis et al. reasoned that a centrally placed nick in the target helix might enhance the binding of CRP. They did note a small, albeit significant, increase in affinity (3-fold); however, they argued that the increased affinity could be explained by the presence of the additional negative charge of the dianionic phosphate produced by cleavage of the phosphodiester bond. This explanation is equally applicable to the observation of Koudelka et al. (1988), since both studies utilized nicks with 5′ phosphates. In view of the absence of an obvious increase in flexibility at nicks, it would be of interest to repeat the two binding experiments, utilizing nicks with 5′ hydroxyl groups, in order to determine

whether the observed increases in affinity are due to simple charge effects rather than to flexibility.

Electrophoretic Behavior of Single-Stranded Gaps in DNA. The principal motivation for the current work was an understanding of the influence of points of increased flexibility on the elctrophoretic behavior of DNA. In some investigations, reduced mobility (gel retardation) is taken as evidence for stable bends, by analogy with the behavior of A-tract DNA; however, this relationship has not been investigated systematically. Moreover, there is no adequate quantitative model for the electrophoretic behavior of either stably bent or flexibly hinged DNA. It is immediately apparent (e.g., Figure 2) that gaps of at least 1 nt are strongly retarded on polyacrylamide gels. Since the reduced mobilities are not strongly phase-sensitive (in contrast to A-tract curvature), a substantial portion of the observed decreases in mobility must be due to increased flexibility. Although the degree of flexibility cannot be quantified from the gel studies, it is clear that gaps of 2–4 nt are more flexible than gaps of 1 nt; for the pyrimidine bridges used in the current study, the flexibility effect appears to saturate after 2 nt (Figures 2, 4, and 5).

The magnitude of the electrophoretic retardation observed in the current study can be used to gauge, at least qualitatively, the extent of flexibility noted by others. For example, Georgiadis et al. (1991) presented gel electrophoretic evidence that O^4-alkylation of thymines increases the flexibility of the alkylated molecules. Moreover, because they observed no phase dependence of the reduced mobilities, their argument for isotropic hyperflexibility would appear to be plausible. Those authors observed only slight reductions in mobility for the O^4-alkylthymine molecules (ca. 5–9% reduction in mobility for a 224-bp molecule possessing 14 alkylated sites). By comparison with reductions of up to 50% for a 230-bp molecule with 10 gaps (Figure 4), the degree of hyperflexibility attendant O^4-alkylation would appear to be quite modest; however, it should be noted that Georgiadis et al. used 4% polyacrylamide gels, whereas 6–12% gels were used in the current study.

Werel et al. (1991) observed reductions of 10–15% in gel mobility for 112-bp fragments having a 1-nt gap at the center of the molecule (12% gel, 4 °C). They ascribed the gel behavior to an increase in flexibility at the gap site, although they did not perform any experiments that would distinguish stable, directional curvatures from increased flexibility. The current study demonstrates that at least part of the observed reduction in electrophoretic mobility observed by Werel et al. is, in fact, due to an increase in flexibility. Those authors went on to make an important point regarding the "missing contact" method for mapping protein–DNA contacts (Brunelle & Schleif, 1987; Chalepakis & Beats, 1989; Hayes & Tullius, 1989). In particular, by analogy with the arguments of Koudelka et al. (1988) and of Giraud-Panis et al. (1992), they argued that the 1-nt gap could alter protein-binding affinities as a consequence of long-range alterations in structure and/or flexibility, in addition to the effects of missing nucleotide contacts.

Finally, Kerppola and Curran (1991) investigated the effects of the binding of the eukaryotic transcription regulatory proteins, Fos–Jun and Jun–Jun, on the structure of their DNA targets. Kerpolla and Curran observed that two different gel methods for estimating the magnitude of the induced bend, namely, the circular permutation analysis (Thompson & Landy, 1988) and the phasing analysis (Zinkel & Crothers, 1987) yielded very different magnitudes for the bend angles. In particular, the authors observed that the angles determined by the permutation analysis were as much as 3 times larger than the same angles determined by the phasing analysis. They concluded that the observed differences were due to induced flexibility in the DNA target sequence. While the data presented by Kerppola and Curran do not provide any direct evidence for or against protein-induced flexibility, it should be noted that the permutation analysis relies on an interpretation of the reptation model for the migration of large DNA molecules through gels (Lumpkin & Zimm, 1982) that is not directly applicable to short DNA molecules. Therefore, the differences in the apparent angles determined by the two methods may be due, at least in part, to this latter effect.

CONCLUSIONS

The current analysis has provided additional evidence that nicks in DNA do not represent points of increased flexibility, in agreement with earlier work. Therefore, enzymes that specifically recognize nicks may do so by examining the helix for points of potential weakness rather than points of hyperflexibility. In contrast to nicks, single-stranded gaps of at least 1 nucleotide residue do represent points of significant hyperflexibility, with this latter effect being manifest through substantial reductions in gel mobility of DNA fragments possessing the gaps. The isolated observation of reduced gel mobility cannot, therefore, be taken as evidence for a static bend.

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