Effects of Bulge Composition and Flanking Sequence on the Kinking of DNA by Bulged Bases†

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ABSTRACT: We recently showed that bulged bases kink duplex DNA, with the degree of kinking increasing in roughly equal increments as the number of bases in the bulge increases from one to four [Hsieh, C.-H., & Griffith, J. D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 4833-4837]. Here we have examined the kinking of DNA by single A, C, G, or T bulges with different neighboring base pairs. Synthetic 30 base pair (bp) duplex DNAs containing 2 single-base bulges spaced by 10 bp were ligated head to tail, and their electrophoretic behavior in highly cross-linked gels was examined. All bulge-containing DNAs showed marked electrophoretic retardations as compared to non-bulge-containing DNA. Regardless of the sequence of the flanking base pairs, purine bulges produced greater retardations than pyrimidine bulges. Furthermore, C and T bulges produced the same retardations as did G and A bulges. Bulged DNA containing different flanking base pairs showed marked differences in electrophoretic mobility. For C-bulged DNA, the greatest retardations were observed with G·C neighbors, the least with T·A neighbors, and an intermediate amount with a mixture of neighboring base pairs. For A-bulged DNA, the retardations were greatest with G·C neighbors, less with T·A neighbors, even less with a mixture of neighboring base pairs, and finally least with C·G neighbors. Thus flanking base pairs affect C-bulged DNA and A-bulged DNA differently, and G·C and C·G flanking base pairs were seen to have very different effects. These results imply an important role of base stacking in determining how neighboring base pairs influence the kinking of DNA by a single-base bulge.

Extra unpaired or bulged bases in double-helical DNA can arise from recombination between sequences that are not fully homologous or from errors in replication. When RNA folds on itself, the duplex segments created frequently contain extra base bulges. Bulged bases resulting from replicative errors are considered to play an important role in frame-shift mutagenesis (Streisinger et al., 1966); indeed, HIV reverse transcriptase adds or deletes bases at a relatively high rate (Preston et al., 1988; Roberts et al., 1988; Weber & Grosse, 1989). Extra bases in duplex segments of RNA may create specific protein recognition sites as shown for the bulged A residue required for the binding of the coat protein of phage R17 to R17 RNA (Wu & Uhlenbeck, 1987). In *Escherichia coli*, the repair of heteroduplex DNAs containing either one (Dohet et al., 1986) or ten (Fishel et al., 1986) extra bases was shown to occur via the methyl-directed mismatch repair pathway. In vitro, this same repair system was found to correct DNA containing 1-3 extra bases as efficiently as a G·T mismatch (Learn & Grafstrom, 1989). It will be important to learn how bulged bases shape the three-dimensional structure of naturally folded RNA and what features of bulge-containing DNA are seen by the specific repair systems. Such an understanding will require information about the effects of each of the four bases individually, and in the context of different flanking sequences since stacking effects of the base pairs adjacent to the bulged base(s) and the different size of purine and pyrimidine bases would be expected to be major determinants.

Single extra bases in short duplex DNAs have been examined in detail by NMR and X-ray crystallographic methods. These studies indicate that the bases may exist either stacked into the helix or in a looped-out conformation. Using NMR spectroscopy, Woodson and Crothers (1988) found that a single extra G in a 10 bp[1] duplex was stacked into the helix. In similar short duplexes (13 and 15 bp), single extra A's were also found to stack into the helix (Patel et al., 1982; Hare et al., 1986; Roy et al., 1987) independently of temperature below the melting point of the duplex and independently of flanking sequences (Kalnik et al., 1989a). Also by use of NMR methods, a single extra T in a 9 bp duplex was observed to stack into the helix (van den Hoogen et al., 1988), while an extra C was seen to loop out or stack in depending on the temperature of the solution (Kalnik et al., 1989b). X-ray crystallographic studies of a 13 and a 15 bp duplex containing an extra A at a single site similar to those examined by NMR methods in solution found the extra A to be looped out of the helix in the crystal, likely reflecting the forces of crystallization (Joshua-Tor et al., 1988; Miller et al., 1988).

We recently utilized EM[1] and gel electrophoresis to examine the effects of 1-4 extra bases in the context of much larger DNAs (Hsieh & Griffith, 1989) as the proximity of the extra bases to the ends of the short duplexes was of concern. In this approach, 32 bp DNA duplexes are synthesized containing unique single-stranded ends such that upon ligation the 32-mers can only join in an oriented head-to-tail fashion. The 32-mers are synthesized to contain various arrangements of single or multiple extra bases. The effects of the extra bases in each 32-mer are amplified by the ligation process which places the extra bases in phase with the helix in the linear and circular multimers. It was observed that single extra A's caused a marked retardation of the electrophoretic mobility of the linear multimers, indicating that the bulged bases bent

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[1] Abbreviations: bp, base pair(s); EDTA, ethylenediaminetetraacetic acid; EM, electron microscopy.

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or kinked the DNA. Analysis of the size distribution of the circular multimers confirmed this observation. DNAs with two to four extra bases at a single site showed even greater electrophoretic retardations and yielded smaller circular multimers. Placing the extra base(s) out of phase with the helix created kinks which canceled each other and produced no net retardation. Using a similar approach, Bhattacharyya and Lilley (1989) examined the effects of single and multiple extra A's or T's and came to similar conclusions. Rice and Crothers (1989) also utilized electrophoresis to examine the effects of a single extra A and concluded that it kinks DNA.

Central questions remain as to the link between the electrophoretic and NMR observations on bulge-containing DNAs. Is there a simple relation between a base stacking into the helix and kinking as assayed by electrophoretic retardation? If so, are DNAs containing looped out bases not kinked, or will they nonetheless show some retardation as contrasted to non-bulge-containing DNA? Answers to these questions should provide a better link between the two methods and add to our understanding of each method individually. Studies of how the electrophoretic retardations depend on the base composition of the bulge and on the sequences flanking the bulge should help link the two methods since, using NMR, Kalnik et al. (1989a) demonstrated that an extra A stacked into duplex DNA independent of flanking sequence and that an extra A flanked by G's (T_m = 48.5 °C) is more stable than one flanked by C's (T_m = 43.5 °C).

There are practical reasons for relating the electrophoretic retardation of bulge-containing DNAs to the nature of the bulge. Recently, a method for detecting small deletions in chromosomal genes has been developed on the basis of polymerase chain reaction (PCR) amplification and the knowledge that bulged bases retard the electrophoretic mobility of DNA. In one such study, PCR products from exon 10 of the cystic fibrosis gene were examined by polyacrylamide gel electrophoresis. Approximately 70% of the mutations that result in cystic fibrosis correspond to a deletion of three base pairs at a specific site in exon 10 (Kerem et al., 1989). A DNA segment containing this site centrally located is amplified by PCR. If the patient is heterozygous for the deletion and thus a carrier, the PCR products when annealed will form two homoduplex and two heteroduplex species. The latter will contain a three-base bulge which will retard the mobility of the DNA upon electrophoresis in highly cross-linked gels (Anglani et al., 1990; Barker, 1990). For this new method to be of general value, it will be essential to understand how the retardation depends on the base composition of the bulged bases and on the nature of the sequences flanking the bulge.

In this study, oligonucleotides containing two one-base bulges separated by 10 bp were synthesized, and these 30 bp duplexes were ligated head to tail in an oriented fashion. Four different bulged bases (A, G, C, or T) placed between four different flanking base pairs (G·C, C·G, T·A, or mixed) were examined and the electrophoretic mobility of the ligated oligonucleotides was compared. The results show that purine bulges produce greater electrophoretic retardations than the pyrimidine bulges regardless of the sequence of the flanking base pairs; A-bulged bases and G-bulged bases produce the same effects and C-bulged DNA behaved like the T-bulged DNA. Moreover, bulged DNA containing different flanking base pairs showed significant differences in electrophoretic mobility. These results suggest that base stacking plays an important role in DNA kinking by extra bases.

Materials and Methods

Oligonucleotide Preparation. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. The crude oligonucleotides were purified by electrophoresis on 20% polyacrylamide–7.5 M urea gels in TBE buffer (89 mM Tris base–89 mM boric acid–2 mM EDTA[1] at pH 8.0). Oligonucleotides were visualized by UV shadowing, eluted from polyacrylamide gels, and recovered by ethanol precipitation.

Preparation and Ligation of Duplex DNAs. DNA duplexes were prepared by heating a mixture containing equal amounts of the two complementary strands at 65 °C for 10 min and then allowing the DNA to cool to room temperature over 4 h. The duplex DNAs were incubated with 2 units of T4 DNA kinase (BRL)/μg of DNA at 37 °C for 1 h and purified by chloroform–isoamyl alcohol (24:1 v/v) extraction and ethanol precipitation. For ligation, the phosphorylated DNAs were incubated with T4 DNA ligase (purified in this laboratory) at an enzyme concentration of 3 units/μg of DNA and a DNA concentration of 40 μg/mL for 16 h at 4 °C.

Gel Electrophoresis. The ligated DNAs were electrophoresed on 15% polyacrylamide gels [30:1 acrylamide:bis(acrylamide) ratio] in TBE buffer at room temperature for 5 h at 12 V/cm. DNA was visualized by staining with ethidium bromide and photographed under UV illumination.

Results

The oligonucleotides that were synthesized for these experiments contain two one-base bulges (in all cases the same base for each 30-mer) separated by 10 bp and single-stranded ends such that the 30 bp duplexes can join only in a head-to-tail arrangement. This design places the bulged bases in phase and amplifies the effect of bulged bases in the multimeric DNA (Hsieh & Griffith, 1989). The 30 bp monomers were designed with two one-base bulges to give greater effects on gel mobility than would be obtained by a single one-base bulge, and three one-base bulges would place two of them too close to the ends of the duplex. Figure 1 shows the sequence of these DNAs and a control DNA containing no bulged bases; DNAs with the four possible bulged bases were prepared. Furthermore, for each bulged base, DNAs with bulges flanked by G·C, or C·G, or T·A base pairs on both sides, or a species in which the bulges were flanked by a mixture of base pairs was synthesized (see Figure 1). This set of DNAs makes it possible to compare the relative kinking of DNA by the four different bulged bases and in the context of different flanking base pairs.

DNA Kinking by Different Bulged Bases. The DNA duplexes in Figure 1 were ligated head to tail, and their mobilities in 15% polyacrylamide gels were examined. The gels were photographed and the position of each monomer and multimer band was determined relative to *Hae*III-digested *φX174* DNA standards. A plot of these data (the *k* value defined as the ratio of the apparent size of a DNA fragment to its actual size) representing the relation between the apparent size of the DNA in each band and its known size provides a clear measure of the degree of kinking of each of the bulge-containing duplexes; here families of 30-mers that are retarded due to kinking are represented as a line of greater slope. All DNAs containing bulged bases showed marked retardation of mobility as compared to the control DNA (Figures 2, 3, and 5). DNAs containing bulged A's showed greater retardations than DNA containing bulged C's irrespective of the flanking base pairs (see Figure 2, lanes 3 and 4 flanked by G·C base pairs, lanes 5 and 6 flanked by T·A base pairs, and lanes 7 and 8 flanked by mixed base pairs).

The difference in retardations due to bulged A's or C's may reflect the difference in size between the purines and pyrimidines or base-specific effects. To examine this, the elec-

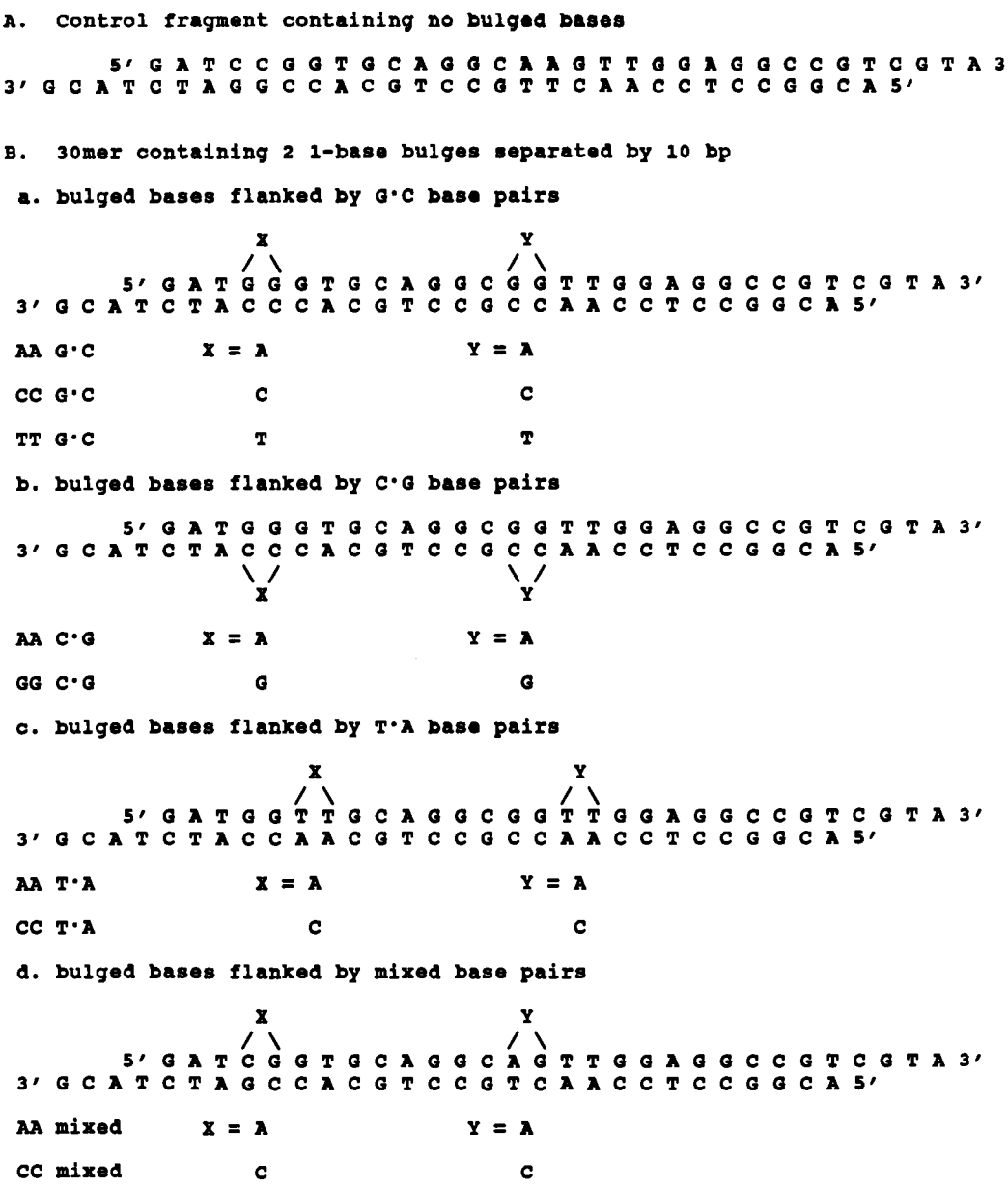

FIGURE 1: Sequences of synthetic duplexes. (A) The duplex containing no bulged bases. (B) Duplexes containing two one-base bulges. The 30 bp duplexes were designed with two one-base bulges to give greater effects on gel mobility than would be obtained by a single one-base bulge. The name for each duplex is indicated on the left.

trophoretic mobilities of four duplexes were compared. In two (termed CC G·C and TT G·C), each duplex contained two single bulged C's or T's spaced by 10 bp and flanked by G·C neighbors, and in the other two (termed AA C·G and GG C·G), two single A's or G's were flanked by C·G neighboring base pairs (the latter neighbors were chosen to avoid slippage of the extra G). Figure 3 shows that the TT G·C duplex exhibited a degree of retardation similar to the CC G·C duplex (Figure 3, lanes 4 and 5; Figure 4) and that the GG C·G duplex behaved like AA C·G (Figure 3, lanes 6 and 7; Figure 4). Thus bulged purines produce greater retardations than bulged pyrimidines, and the effects of A and G are equal as are the effects of C and T.

The Role of Flanking Base Pairs on DNA Kinking by Bulged Bases. In Figures 2 and 3, it can be seen that DNAs containing the same bulged bases but having different flanking base sequences show different degrees of retardation. For example, in lanes 3, 5, and 7 of Figure 2, DNAs containing the same bulged bases (A) but flanked by different base pairs (G·C, T·A, or mixed) have very different electrophoretic mobilities. The effect of the flanking base sequences on DNA structure is particularly clear in Figures 5 and 6. Here, DNAs each with bulged C's but having different flanking base pairs were seen to behave differently, the retardation being greatest with G·C neighbors, next with mixed neighbors, and least with T·A neighbors (Figure 5, lanes 3–5; Figure 6A). The retardation for the A-bulged DNA followed the order in which the greatest retardation was with G·C neighbors, less with T·A neighbors, less still with mixed neighbors, and finally least with C·G neighbors (Figure 5, lanes 6–9; Figure 6B). As discussed below, these results likely reflect the effects of base stacking on the kinking of bulged DNA as contrasted to base pairing.

Discussion

In this study we have examined the kinking of DNA by single-base bulges of different composition and different neighboring base pairs. Synthetic 30 bp duplex DNAs containing two single-base bulges spaced by 10 bp were ligated head to tail and their electrophoretic behavior in highly cross-linked gels was examined. All bulge-containing DNAs showed marked electrophoretic retardation as compared to

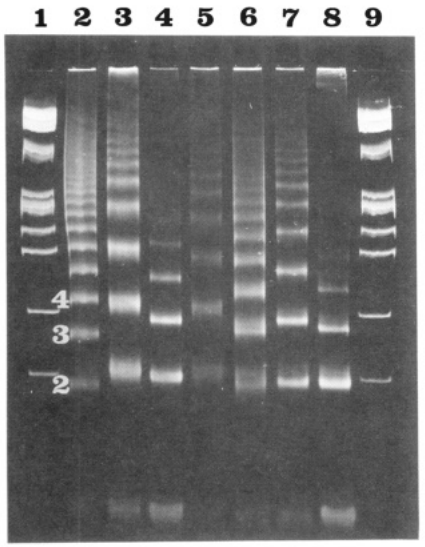

FIGURE 2: Electrophoretic analysis of ligated DNAs containing A- or C-bulged bases flanked by different base pairs. Ligated DNAs (see Figure 1 and Materials and Methods) were electrophoresed through a 15% polyacrylamide gel and visualized by staining with ethidium bromide. Lanes 1 and 9, *Hae*III digest of $\phi X174$ DNA. Lane 2, a control DNA containing no bulged bases (the numbers indicate dimer, trimer, and tetramer bands). Lane 3, DNA containing A-bulged bases flanked by G·C base pairs (AA G·C). Lane 4, DNA containing C-bulged bases with G·C flanking sequences (CC G·C). Lanes 5 and 6, DNA containing A-bulged (AA T·A) and C-bulged bases (CC T·A), respectively; both were flanked by T·A base pairs. Lanes 7 and 8, DNA containing A-bulged (AA mixed) and C-bulged bases (CC mixed), respectively; both were flanked by mixed base pairs.

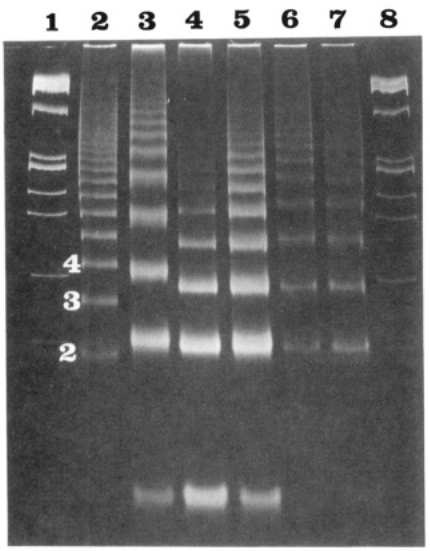

FIGURE 3: Effect of different bulged bases on the electrophoretic mobility of DNA. DNA duplexes were ligated head to tail, electrophoresed through a 15% polyacrylamide gel, and visualized by staining with ethidium bromide. Lanes 1 and 8, *Hae*III digest of $\phi X174$ DNA. Lane 2, a control DNA with no bulged bases (the numbers indicate dimer, trimer, and tetramer bands). Lanes 3–5, DNA containing A-bulged (AA G·C), C-bulged (CC G·C), and T-bulged bases (TT G·C), respectively; all bulged bases were flanked by G·C base pairs. Lanes 6 and 7, DNA with A-bulged (AA C·G) and G-bulged bases (GG C·G), respectively; both were flanked by C·G base pairs.

non-bulge-containing DNA. Regardless of the sequence of the flanking base pairs, purine bulges produced greater retardations than pyrimidine bulges. Furthermore, C and T bulges produced the same retardations as did G and A bulges. Bulged DNA containing different flanking base pairs showed marked differences in electrophoretic mobility, with the

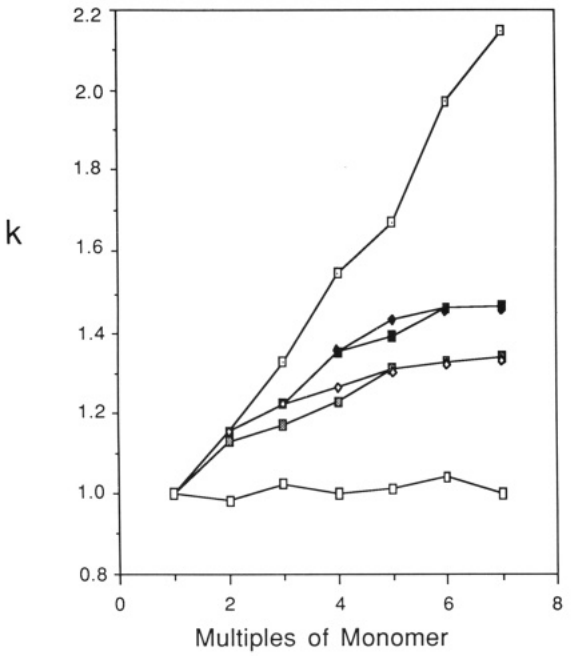

FIGURE 4: Electrophoretic mobility of ligated DNA containing bulged bases. The k value is defined as the ratio of the apparent size of a DNA fragment to its actual size. The apparent size of the DNA in each band in lanes 2–7 of the gel in Figure 3 was determined relative to the mobility of *Hae*III-digested $\phi X174$ DNA fragments (lanes 1 and 8 of Figure 3). These k values were plotted against the known size of the DNA as multiples of the monomer length. (⊡) A-bulged DNA flanked by G·C base pairs (AA G·C); (♦) A-bulged DNA (AA C·G) and (■) G-bulged DNA (GG C·G), both flanked by C·G base pairs; (◊) T-bulged DNA (TT G·C) and (stippled squares) C-bulged DNA (CC G·C), both flanked by G·C base pairs; (□) a control DNA with no bulged bases.

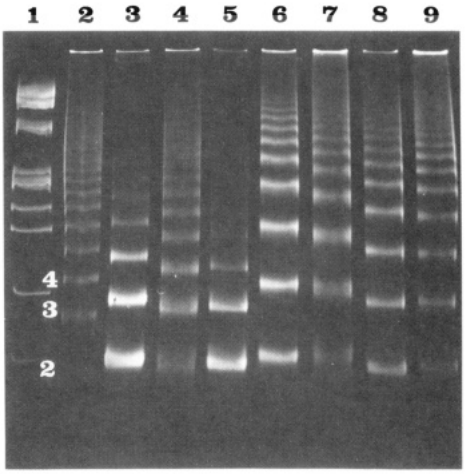

FIGURE 5: Effect of the flanking sequences on the electrophoretic mobility of bulge-containing DNA. Ligated DNA was electrophoresed through a 15% polyacrylamide gel and visualized by staining with ethidum bromide. Lane 1, *Hae*III digest of $\phi X174$ DNA. Lane 2, a control DNA with no bulged bases (the numbers indicate dimer, trimer, and tetramer bands). Lanes 3–5, C-bulged DNA flanked by G·C (CC G·C), T·A (CC T·A), and mixed base pairs (CC mixed), respectively. Lanes 6–9, A-bulged DNA flanked by G·C (AA G·C), T·A (AA T·A), mixed (AA mixed), and C·G base pairs (AA C·G), respectively.

flanking base pairs affecting C-bulged DNA and A-bulged DNA differently. These results argue for an important stacking contribution in determining the degree to which a single-base bulge will kink duplex DNA.

Using an approach similar to that applied here, we recently showed that bulged bases kink duplex DNA and that the degree of kinking increases in roughly equal increments as the

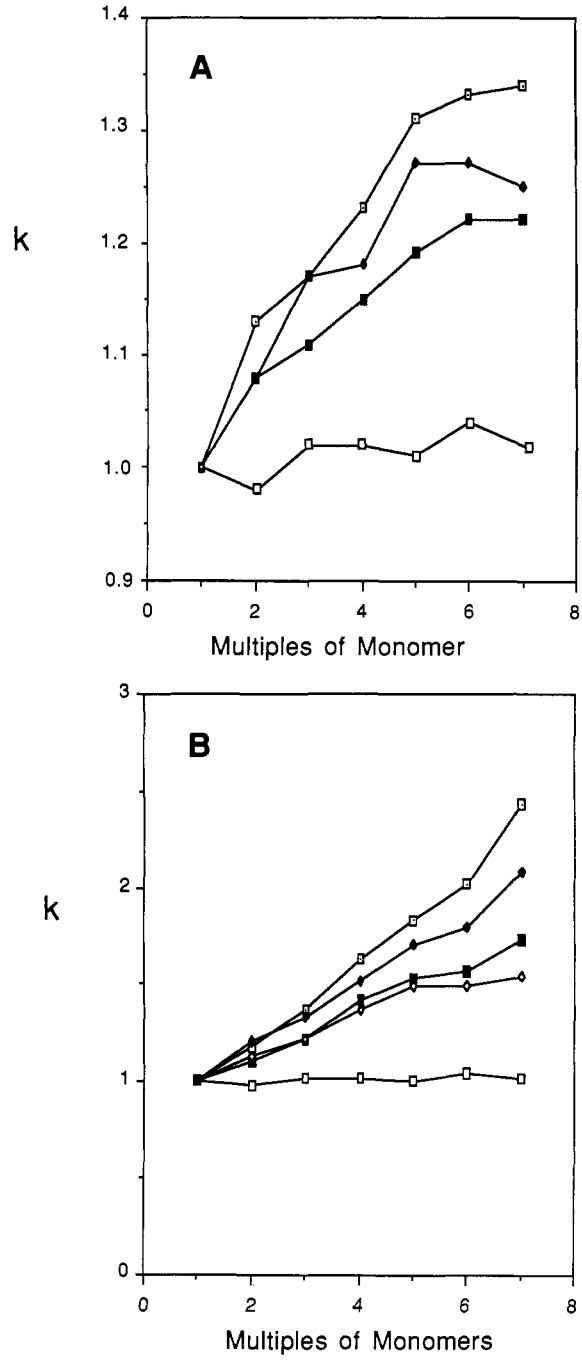

FIGURE 6: Electrophoretic mobility of DNA containing bulged bases flanked by different base pairs. A relative mobility plot was prepared from the DNA in lanes 2–9 of Figure 5 as in Figure 4. (A) DNA containing C-bulged bases: (⊡) flanked by G·C base pairs (CC G·C); (♦) flanked by mixed base pairs (CC mixed); (■) flanked by T·A base pairs (CC T·A); (□) a control DNA with no bulged bases. (B) DNA containing A-bulged bases: (⊡) flanked by G·C base pairs (AA G·C); (♦) flanked by T·A base pairs (AA T·A); (■) flanked by mixed base pairs (AA mixed); (◊) flanked by C·G base pairs (AA C·G); (□) a control DNA containing no bulged bases.

number of bases in the bulge increase from 1 to 4 (Hsieh & Griffith, 1989). In that study, the bases in the bulges and the flanking base pairs were a mixture so that no conclusions could be drawn about the relative degree of kinking by any single base or within different sequence environments. Here we have addressed this problem with a much more complete description of DNA kinking for single-base bulges. Bhattacharyya and Lilley (1989) recently reported that A bulges produce a greater electrophoretic retardation than T bulges and that the flanking base pairs will affect the degree of kinking by A bulges. However, they did not examine C or G bulges, and they failed to observe any influence of flanking sequence for T-bulged DNA. Our results show that the effect of flanking sequence is small for the pyrimidine base bulged DNA (Figure 6A) compared to the purine base bulged DNA (Figure 6B). By ligating the short bulge-containing DNAs head to tail, we have amplified the effects of any single lesion and thus made it possible to observe smaller effects than what can be detected by examination of the DNA monomers alone as in the study of Bhattacharyya and Lilley (1989). Rice and Crothers (1989) also utilized electrophoresis to examine kinking of DNA by single A- or T-bulged bases and reported similar observations.

Our results show that purine-bulged bases produce greater electrophoretic retardations than pyrimidine bases. The simplest explanation is that the bulge-induced kinks are relatively static, with the kinking angle being greater for the purine bases due to their greater size. A more complex argument would be that the purines stack into the helix kinking the DNA and that they remain stacked in while the pyrimidines oscillate between a stacked in and looped out conformation. In this model the difference in electrophoretic retardations of the purines and pyrimidines would be attributed to the lesser time that the bulged pyrimidines exist in a stacked in state rather than a difference in the size of the bases. From NMR studies it was observed that bulged A's stack into the helix independently of temperature (Kalnik et al., 1989a), whereas extra C's appear to stack in at 40 °C and loop out at 0 °C (Kalnik et al., 1989b). If the latter model was correct, it would be expected that DNAs containing A bulges and C bulges would show similar electrophoretic retardations at 40 °C where both bases would be stacked in. However, in other studies (data not shown) we have observed that A bulges produce greater retardations than C bulges at 40 °C. Moreover, also using NMR, van den Hoogen et al. (1988) observed that an extra T remained stacked into the helix irrespective of temperature, and our results show that T-bulged DNA produces a different retardation pattern than A-bulged DNA. Thus the simple explanation in which the larger size of the purine bases produce a greater angle of kinking together with secondary effects of neighboring base pairs on stabilizing the kink appears to provide the best model.

Rice and Crothers (1989) have argued that an extra adenine base underwinds DNA by 0.7 bp and increases the number of base pairs per helical turn. Therefore, differerent degrees of unwinding could place bulged bases out of phase and produce different degrees of distortion from the planar bend which might cause differences in gel mobility. Our data presented here cannot completely rule out this possibility. However, were the differences observed here between, for example, A-bulged DNAs and C-bulged DNAs due to the result of different degrees of unwinding by the bulged base, then we would not expect the two respective lines in the K plots to follow each other uniformly as they do. Rather, phasing effects should vary greatly with the DNA size and would produce lines that would oscillate relative to each other. Furthermore, we have observed that in the assay for detecting cystic fibrosis carriers the two heteroduplex species (a AAG bulge and a CTT bulge) have different mobilities in polyacrylamide gels, with the AAG-bulged DNA being retarded more than the CTT-bulged DNA as predicted from this study. Here there is only a single bulge in each DNA and questions of phasing do not apply. These observations argue against a major contribution of unwinding in gel retardation of bulge-containing DNA. Thus different gel mobilities among these DNA duplexes are mainly attributed to different degrees of kinking caused by bulged

bases under the influence of flanking base pairs.

Bulged bases can be found in DNA as a result of recombination or of errors in replication. Transfection experiments with heteroduplex DNA containing either one (Dohet et al., 1986) or ten (Fishel et al., 1986) bulged bases indicated that these lesions are repaired by the methyl-directed mismatch repair pathway. Using an in vitro DNA repair assay, Learn and Grafstrom (1989) demonstrated that the methyl-directed mismatch repair pathway can repair heteroduplexes containing one to three bulged bases as efficiently as a G·T mismatch. We have shown here that different bulged bases, combined with various flanking base pairs, produce different perturbations in the DNA helix. In the future it will be important to correlate the degree of kinking of DNA by combinations of bulged bases and their neighboring base pairs with the relative rates of their correction by these repair systems.

Bulged bases also occur when RNA folds on itself to create complex secondary and tertiary structures and the bulges serve as junctions that connect the adjacent duplex segments in folded RNA. Moreover, bulges in RNA molecules may serve as specific protein recognition sites. Recently, the properties of bulged bases in both RNA and RNA–DNA duplexes were examined by gel electrophoresis (Bhattacharyya et al., 1990). For both types of duplexes, the extent of electrophoretic retardation was greater for adenine than for uracil bulges for a particular size of the bulge. If the results presented here hold true for RNA, they will be useful for predicting the tertiary structures of RNA molecules. For example, junctions containing bulged bases between two duplex segments would produce kinks that would hold the two duplex segments at precise angles relative to each other; the angle would depend on the number of extra bases, their composition, and the neighboring base pairs.

Registry No. AA G·C, 131010-00-9; CC G·C, 131010-01-0; TT G·C, 131010-06-5; AA C·G, 131010-02-1; GG C·G, 131010-03-2; AA T·A, 131010-04-3; CC T·A, 131010-05-4; AA mixed, 131009-97-7; CC mixed, 131009-98-8; A, 73-24-5; G, 73-40-5; C, 71-30-7; T, 65-71-4; control fragment containing no bulged bases, 131009-99-9.

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