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Deletions of bases in one strand of duplex DNA, in contrast to single-base mismatches, produce highly kinked molecules: Possible relevance to the folding of single-stranded nucleic acids

(RNA structure/electrophoretic migration/electron microscopy)

CHENG-HSILIN HSIEH AND JACK D. GRIFFITH

Lineberger Cancer Research Center and Department of Microbiology and Immunology, University of North Carolina, Chapel Hill, NC 27514

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ABSTRACT **A 32-base-pair (bp) DNA duplex with deletions in one strand, and thus extra bases in the opposing strand, was ligated head-to-tail to produce linear and circular multimers. The electrophoretic mobility of the linear multimers was analyzed in polyacrylamide gels and the size of the circular DNA was determined by electron microscopy. A 1-base deletion produced a marked retardation in the mobility of the linear multimers coincident with the formation of a population of multimeric circles of a smaller average size than the deletionless 32-mer; 2-, 3-, or 4-base deletions at the same site produced proportionately greater effects. Two 1-base deletions separated by 10 bp on the same strand produced a greater reduction in mobility than a 1-base deletion, whereas two 1-base deletions spaced by 5 bp on the same strand yielded a molecule that behaved more like the deletionless DNA. We conclude that deletions of 1–4 bases at a single site on duplex DNA produce molecules that behave as if they contain sharp bends or kinks. In contrast, single mismatches in the 32-bp duplex produced no abnormality in behavior relative to normally base-paired DNA in the gel mobility and electron microscopic assays. The possible role of such structures in organizing the three-dimensional folding of single-stranded nucleic acids is considered.**

Extra bases in one strand of double-stranded DNA or RNA may result from a variety of natural mechanisms. When single-stranded RNA or DNA folds on itself, the resulting secondary structure can be thought of as a chain of duplex rods containing perfectly and imperfectly paired bases linked by junctions containing extra bases in one of the two opposing strands. How these junctions contribute to the overall folding of single-stranded DNA or RNA is not established, but were they to provide a precise orientation to the adjoining duplex segments, a possibility raised here, an understanding of their structure would be very important—in particular to our knowledge of the folding of natural RNA.

Extra or deleted bases can also be found in duplex DNA as a result of recombination or of errors in replication. *In vitro*, the RecA protein of *Escherichia coli* readily catalyzes recombination reactions between two DNAs that differ from each other by a number of bases, giving rise to recombinant heteroduplexes containing base deletions in one strand (1, 2). *In vitro* studies have suggested how the deletion of bases during replication could lead to frameshift mutations (3), and *in vivo*, bacteriophage T4 DNA polymerase plays a central role in controlling the frequency of frameshift mutations (4). Deletions or insertions of bases in one strand can result from DNA synthesis *in vitro* where, in general, single-base deletions predominate and most often occur in runs of the same nucleotide (5–7). In *E. coli*, the repair of deletions of up to 10 bases in one strand of DNA is carried out by the methyl-directed repair system, which also corrects mismatches in DNA (ref. 8, reviewed in ref. 9). To understand how base deletions or insertions are detected and repaired, it would be valuable to know how they alter DNA structure.

Previous structural studies of extra or deleted bases have focused on whether single extra bases stack into the helix or loop out. These studies used 9- to 15-base oligonucleotides that were nearly self-complementary so that the annealing of two molecules produced a short DNA duplex with 2 single extra bases. NMR methods have been used to examine the structure of several 13- and 15-mers of this form containing single extra adenosines. The adenosines were found to stack into the helix (10–12) independently of temperature below the melting point of the duplex and independently of whether they were flanked by cytosines or guanosines (13). However, when one of these DNAs was crystallized it appeared that the extra adenosines were forced out of the helix (14, 15). The latter result may reflect the forces of crystal formation, since examination of similar short duplexes with extra cytosines by NMR methods in solution indicated that the cytosines will loop out or stack into the helix depending on temperature (16, 17).

In the two-dimensional NMR studies (11, 12), models were presented in which the extra bases stack into the helix to produce a wedge that kinks the DNA. However, whether extrahelical, looped-out bases would also kink the DNA was not addressed. Thomas *et al.* (18) noted that a 3-base deletion in one strand of a 268-base-pair (bp) fragment produced a marked retardation in its mobility in 5% polyacrylamide gels whereas a single-base mismatch did not, suggesting that the 3-base loop produced a kinked molecule. These studies prompted us to apply electrophoretic and electron microscopic methods to examine in more detail the effects of deletions in one strand of duplex DNA.

In the studies reported here, a series of 32-bp oligodeoxynucleotides was synthesized based on a DNA sequence used in a previous study (19). Deletions were introduced at one or two sites on one strand of the 32-mer, producing molecules with the deletions separated by 0.5 or 1 helical turn. Unique single-stranded ends of the 32-bp duplex allowed the molecules to be oriented head-to-tail upon ligation, placing the deletion(s) 3.0 helical turns apart and multiplying the effect of each deletion in the multimeric DNA. The results were examined by following the electrophoretic mobility of linear multimers and the distribution of circle sizes as determined by electron microscopy (EM). The results provide direct evidence for deletions of 1–4 bases in one strand of DNA producing a sharp kink, consistent with the models drawn from the NMR data (11, 12).

Large changes in the physical structure of DNA containing single-base mismatches have not been observed (reviewed in ref. 9); however, since the *E. coli* methyl-directed repair system corrects both mismatches and short deletion/insertion lesions (8, 9), it seemed worthwhile to examine the effects of single-base mismatches in the 32-mer by the gel

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mobility and EM assays. No significant abnormality in behavior relative to normally base-paired DNA was detected.

MATERIALS AND METHODS

Preparation of the DNAs. Oligonucleotides were synthesized on an Applied Biosystems model 380B DNA synthesizer. The crude oligonucleotides were separated by 20% polyacrylamide/7.5 M urea gel electrophoresis, phosphorylated with T4 polynucleotide kinase (Bethesda Research Laboratories), and finally purified by chloroform/isoamyl alcohol (24:1, vol/vol) extraction and ethanol precipitation. Duplexes were prepared by annealing equal amounts of the two complementary strands by heating at 65°C for 10 min and then allowing the DNA to cool to room temperature over 2 hr.

Ligation of the DNAs and Electrophoresis. The duplex oligonucleotides (21-mer or 32-mers) were ligated at a DNA concentration of 40 μg/ml for 16 hr at 16°C, using 3 units of T4 DNA ligase (purified in this laboratory) per μg of DNA. For electrophoresis, the ligated DNAs were loaded onto a 15% polyacrylamide gel (30:1 acrylamide/*N,N'*-methylenebisacrylamide weight ratio). The buffer was 89 mM Tris base/89 mM boric acid/2 mM EDTA at pH 8.0. Electrophoresis was at room temperature for 5 hr at 12 V/cm.

EM. Following ligation of the 32-mers, aliquots of the DNA were purified by treatment with 1% sodium dodecyl sulfate for 10 min at 65°C followed by chromatography over AcA 34 resin (LKB) equilibrated with 10 mM Tris acetate, pH 7.5/0.5 mM EDTA. The fractions containing the DNA were pooled and prepared for EM as described (19); in brief, the DNA was mixed with a buffer containing 0.15 M NaCl and 2 mM spermidine, adsorbed to glow-charged copper mesh grids covered by a thin carbon film, washed with water and a graded ethanol series, air-dried, and rotary shadowcast with tungsten in a fully cryopumped evaporator at 10^{-7} torr (1 torr = 133 Pa). Micrographs were taken on a Philips EM400TLG instrument at 20 kV. The circle lengths were measured on photographic prints by using a Summagraphics digitizer coupled to an IBM PC computer driven by software developed in this laboratory.

RESULTS

Electrophoretic Analysis of the Linear Ligation Products of 32-mers Containing Deletions. For these studies we synthesized a series of 32-bp DNAs similar to one used previously (19) but with single-stranded ends that produce a unique head-to-tail orientation upon ligation. Fig. 1*A* shows the sequence of this DNA and its variants. Four 32-mer variants have 1–4 bases deleted in one strand at a single site, three have two 1-base deletions separated by 5 or 10 bases on one strand or by 10 bases but on opposite strands, and one has two 2-base deletions separated by 10 bases on the same strand.

Circularization of the 32-mers to form very small circles requires an accurate alignment of the DNA ends (20). Because the helix repeat in solution is close to 10.5 bp per turn (21, 22), if very small circles are to be formed, it would be ideal if the deletions in the 32-mers with two deletions were separated by 10.5 bp rather than 10 bp. To approximate this, one of the deletions eliminated one of a pair of thymines so that the opposing extra adenine could shift back and forth giving, on the average, 10.5-bp separation for molecules with two 1-base deletions separated by ostensibly 10 bp (Fig. 1).

The 32-mer and variants were ligated head-to-tail and their mobility in 15% polyacrylamide gels was examined. A 1-base deletion in the 32-mer produced a marked retardation in the mobility of the linear multimers as contrasted to the deletionless 32-mer (Fig. 2, lanes 1 and 2). With 2–4 bases deleted at a single site on one strand, even greater mobility retardations were observed (Fig. 2, lanes 3–5). For the variant with 4 bases deleted at a single site, the linear trimer migrated at the same rate as the octamer of the deletionless 32-mer. The apparent length of the DNA in each band in the gel was determined relative to the mobility of *Hae* III-digested φX174 double-stranded DNA fragments (Fig. 2, lane 12) and these values were plotted against their length in base pairs (shown as multiples of monomer length in Fig. 3*A*). This revealed that, for each ladder of bands, there was a linear relation between the true length and the apparent length, with the slope of the line being 1.0 for the deletionless 32-mer and for a different deletionless 32-mer (Fig. 1*C* and Fig. 2, lane 11). With increasing numbers of bases deleted at a single site the slope of the lines increased monotonically with even increments from 1 to ≈2.5 for the DNA with a 4-base deletion.

The mobility properties of the ligated 32-mers with two 1-base deletions separated by 5 bp or 10 bp (in fact 10–11 bp, as described above, but denoted as 10) and the 32-mer containing two 2-base deletions separated by 10 bp are shown in Figs. 2 and 3*B*. The migration of the DNAs was what would be expected if the base deletions created a stiff kink: the ligated 32-mers with two 1-base deletions separated by 5 bp on the same strand, or separated by 10 bp but on opposite strands, behaved like the ligation products of the deletionless 32-mer, whereas the two 1-base or 2-base deletions separated by 10 bp

A 32-mer and variants

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      5'G A T C C G G T G C A G G C A A G T T G G A G G C C G T C G T A3
3'G C A T C T A G G C C A C G T C C G T T C A A C C T C C G G C A5'
  1       5         10        15        20        25        30
                                    X
                                  X X
                                X X X
                              X X X X
                        X           X
                  X                 X                    X X
                        X X         X
                  X (top)           X
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B 21-mer containing the *C. fasciculata* element

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      5'A A A A A T A G C G A A A A A A T G G C C3'
         3'T T T A T C G C T T T T T T A C C G G T T5'
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C Different deletionless 32-mer

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   5'A C G T A C G T C C C A C G T A C G T A A C G T C C A C G T A A3'
3'T T T G C A T G C A G G G T G C A T G C A T T G C A G G T G C A5'
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FIG. 1. (*A*) Synthetic 32-mer and variants. All deletions (X) were in the bottom strand, producing extra bases in the top strand, except for one variant in which one deletion was in the top strand. The variants had one 1-base (thymine at position 19, T^{19}), 2-base (G^{18},T^{19}), 3-base (C^{17},G^{18}, T^{19}), or 4-base (C^{16},C^{17},G^{18},T^{19}) deletions; two 1-base deletions spaced by 5 bp (C^{13},T^{19}); two 1-base deletions spaced by 10 bp (G^{8},T^{19}); two 2-base deletions spaced by 10 bp (A^{12},C^{13},C^{24},C^{25}); or two 1-base deletions spaced by 10 bp but on opposite strands (T^{19}, C^{8} on top strand). (*B*) The 21-mer containing the *Crithidia fasciculata* bent-helix sequence. (*C*) Different 32-mer.

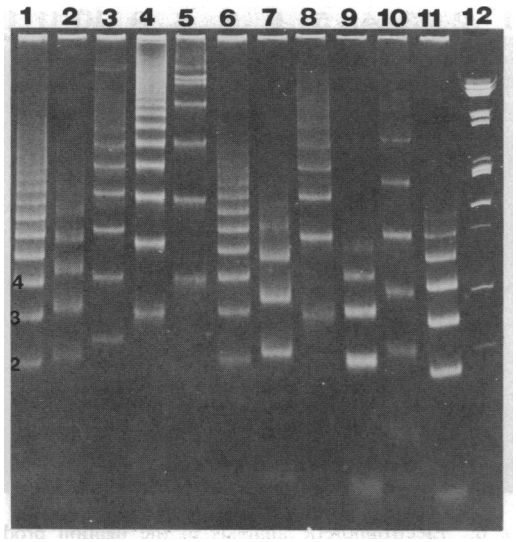

FIG. 2. Electrophoretic analysis of ligated DNAs containing deletions. Duplex oligonucleotides (see Fig. 1) were ligated head-to-tail, electrophoresed through a 15% polyacrylamide gel, and visualized with ethidium bromide. Lane 1, deletionless 32-mer (the numbers indicate dimer, trimer, and tetramer bands); lanes 2–5, 32-mer with one 1-base, 2-base, 3-base, or 4-base deletion, respectively; lane 6, 32-mer with two 1-base deletions spaced by 5 bp; lane 7, 32-mer with two 1-base deletions spaced by 10 bp; lane 8, 32-mer with two 2-base deletions spaced by 10 bp; lane 9, 32-mer with two 1-base deletions spaced by 10 bp but on opposite strands; lane 10, 21-mer containing the *C. fasciculata* bent-helix repeat; lane 11, different deletionless 32-mer; lane 12, *Hae* III digest of ϕX174 duplex DNA.

on the same strand resulted in greater mobility retardations. The retardation of the ligated 32-mer containing a 2-base deletion was similar to that of the ligated 21-mer containing the highly bent *C. fasciculata* motif of phased blocks of 5–6 adenines (ref. 23; Figs. 1*B*, 2, and 3*A*). All ligations were carried out under exactly the same conditions of DNA concentration and time, and no observable changes were noted when the DNA concentration was lower by a factor of 2.

EM Analysis of the Circular Ligation Products of the 32-mers Containing Deletions. Circular DNAs did not enter the 15% polyacrylamide gels, and a significant amount of DNA can be seen at the top of the gel in Fig. 2. To analyze the size distribution of the circles produced by ligation, portions of the ligation mixtures were saved, and the DNA was purified and prepared for EM (Fig. 4). From the micrographs we estimated that ≈25% of the total DNA was circular. Fields of molecules were photographed, the length of each circle was measured, and the distribution of sizes was plotted (Figs. 4 and 5). A 1-base deletion in the 32-mer produced circles with a markedly smaller mean size than the deletionless 32-mer (Fig. 5*A*). Increasing numbers of bases deleted at a single site produced circle distributions shifted toward progressively smaller sizes. For the 32-mer with 3 or 4 base deletions at a single site, nearly half of the circles were 96-bp trimers (Figs. 4 and 5*A*). Analysis of the circular fragments containing two 1-base or 2-base deletions separated by 5 or 10 bp (Fig. 5*B*) was in excellent agreement with the conclusions derived from the electrophoretic analysis.

Electrophoretic and EM Analysis of 32-mers Containing Single-Base Mismatches. Eight 32-mers were synthesized, each containing a different single-base mismatch (Fig. 6 legend). These DNAs were ligated exactly as above and the mobility of the linear multimers in 15% polyacrylamide gels was examined. No shift in the ladder of bands was observed for any of the mismatch-containing DNAs as compared to the DNA with no mismatches (Fig. 6). The distribution of circle sizes following ligation was determined by EM for the 32-mers containing single G/G, C/T, and C/A mismatches, three mispairings believed to have strong, moderate, and weak effects, respectively, on disrupting the helix (9, 24–26). No differences were detected (Fig. 7).

DISCUSSION

In this study we used gel electrophoresis and EM to examine how deletions of a few bases in one strand of a DNA duplex may bend or kink the DNA. Synthetic 32-bp DNAs containing deletions were ligated head-to-tail and the effects of the deletions on the relative electrophoretic mobility of linear multimers and the size distribution of circular multimers were examined. A 1-base deletion produced a marked retardation in the mobility of the ligated multimers coincident with the formation of a population of multimeric circles of a smaller average size than the deletionless 32-mer; deletion of 2, 3, or 4 bases at the same site produced proportionately greater effects. Two 1-base deletions separated by 10 bp on the same strand produced a greater reduction in mobility than a 1-base deletion, while two 1-base deletions spaced by 5 bp on the same strand yielded a molecule that behaved more like the

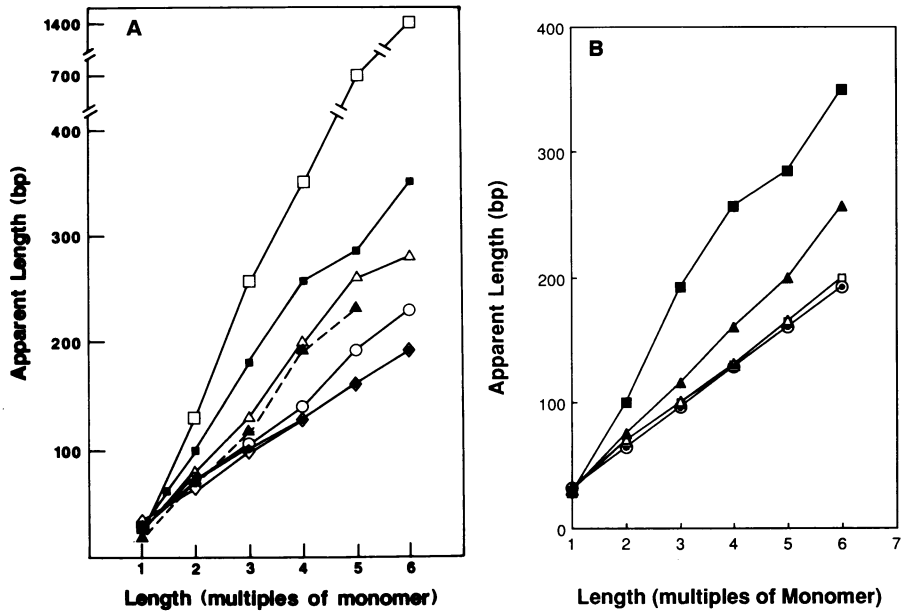

FIG. 3. Relative electrophoretic mobility of ligated oligomers containing base deletions. The apparent length of the DNA in each band in lanes 1–11 of the gel in Fig. 2 was determined based on the migration of ϕX174 duplex DNA fragments (*Hae* III digest) and was plotted against the known length in multiples of the monomer. (*A*) ●, Deletionless 32-mer; ◇, different deletionless 32-mer; ○, 32-mer with a 1-base deletion; △, 32-mer with a 2-base deletion; ■, 32-mer with a 3-base deletion; □, 32-mer with a 4-base deletion; ▲, 21-mer containing the *C. fasciculata* bent-helix repeat. (*B*) ● and ○, deletionless 32-mers as in *A*; □, 32-mer with two 1-base deletions spaced by 5 bp; △, 32-mer with two 1-base deletions spaced by 10 bp but on opposite strands; ▲, 32-mer containing two 1-base deletions spaced by 10 bp; ■, 32-mer containing two 2-base deletions spaced by 10 bp.

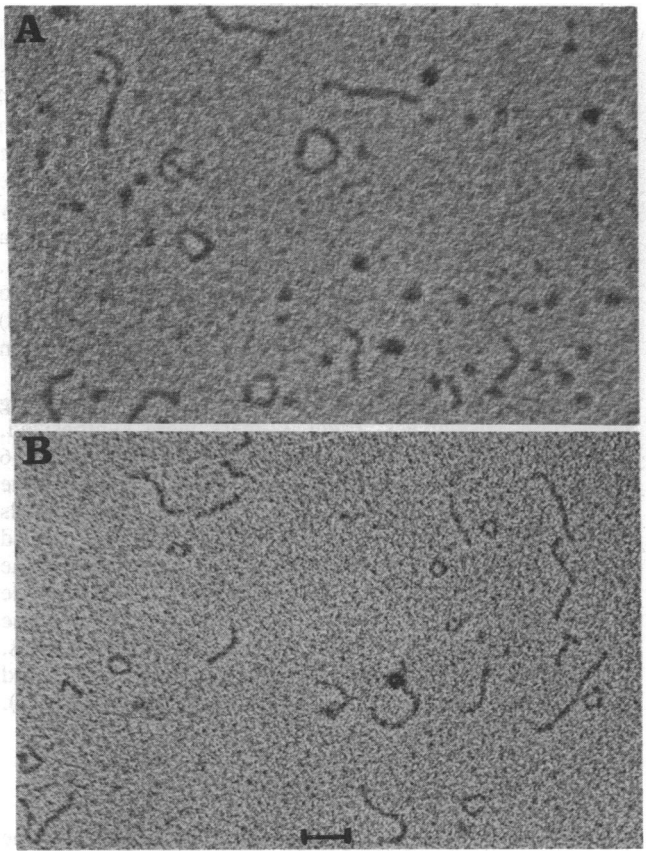

FIG. 4. EM visualization of circular multimers of the ligated 32-mers. The ligation products of the deletionless 32-mer (*A*) and the 32-mer with one 4-base deletion in one strand (*B*) were prepared for EM by direct adsorption to thin carbon films and rotary shadow-casting with tungsten. The two preparations were shadowcast at different times, resulting in thicker-appearing strands in *A*. (Bar = 100 bp.)

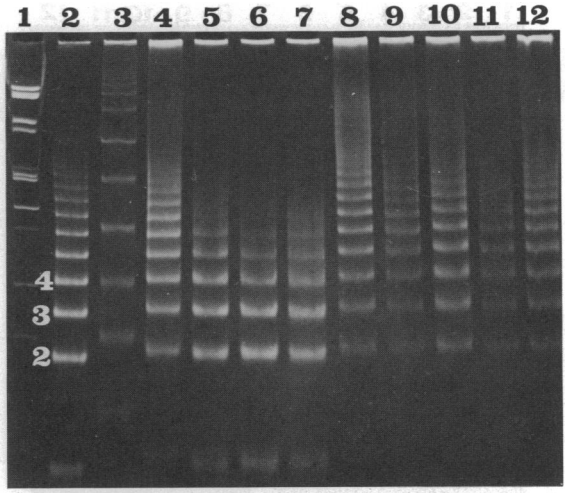

FIG. 6. Electrophoretic analysis of the ligation products of 32-mers containing single-base mismatches. Duplex oligonucleotides were ligated head-to-tail and electrophoresed through a 15% polyacrylamide gel. Lane 1, *Hae* III digest of ϕX174 duplex DNA; lane 2, 32-mer of a different sequence (Fig. 1*C*) from the one containing the mismatches (numbers indicate dimer, trimer, and tetramer bands); lane 3, 21-mer containing phased blocks of adenines (*C. fasciculata* element; Fig. 1*B*); lane 4, 32-mer (Fig. 1*A*) lacking mismatches; lanes 5–12, 32-mers as in lane 4 but with single-base mismatches at position 14 as follows (the first letter corresponds to the top strand, the second to the bottom strand): lane 5, G/G; lane 6, G/T; lane 7, T/T; lane 8, A/G; lane 9, A/A; lane 10, C/C; lane 11, T/C; lane 12, A/C.

deletionless DNA. These results are best explained by the presence of stiff kinks in the DNA at the sites of the deletions.

Our data argue against two possible models: (*i*) that the deletions create bulges on the side of the DNA that do not kink the DNA and (*ii*) that the deletions create flexible joints. The results of the in-phase and counter-phase experiments with 1-base deletions are exactly what would be predicted from the presence of a stiff kink, as seen by gel retardation and circle size distribution measurements. If the deletions create extrahelical loops or bulges that do not kink the DNA, then larger and larger bulges would most likely produce greater and greater gel retardations, a result that was observed. However, *a priori*, two such bulges should cause an equal retardation whether they are spaced by 5 or 10 bp along the DNA, a result that was not observed. Furthermore, bulges that do not kink the DNA would not be expected to reduce the mean circle size distribution over that of the deletionless 32-mer unless they also produce flexible joints, yet a diminution in circle size was observed. Were the deletions to create flexible joints, such joints would compact the DNA and probably reduce its mobility in gels. However, as above, this effect should be additive whether the flexible

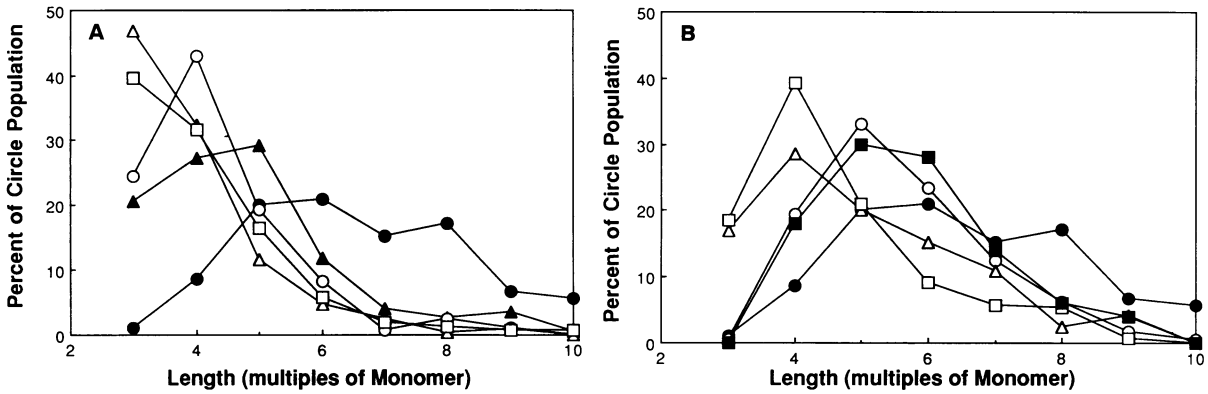

FIG. 5. Distribution of circle sizes produced by the ligation of the 32-mers with and without base deletions. The distribution of circle lengths formed by ligation of the 32-mers was determined directly from the electron micrographs. For each sample 75–100 circles were measured. For each DNA circle the length was determined from the micrograph and then converted to base pairs by using conversion factors (21) that consistently give correct length values for larger DNAs of known size. Each length value was then plotted to the nearest whole multiple of the monomer length. (*A*) ●, No deletion; ▲, 1-base deletion; ○, 2-base deletion; △, 3-base deletion; □, 4-base deletion. (*B*) ●, No deletion; ○, two 1-base deletions on the same strand separated by 5 bp; △, two 1-base deletions on the same strand separated by 10 bp; ■, two 1-base deletions separated by 10 bp but on opposite strands; □, two 2-base deletions on the same strand separated by 10 bp.

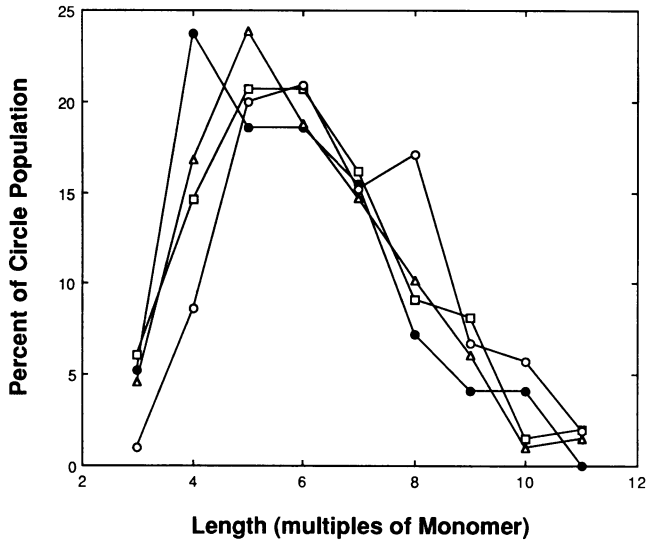

FIG. 7. EM analysis of the distribution of circle sizes of ligated 32-mers containing single-base mismatches. Electron micrographs of the circular DNA forms produced by the ligation of three different 32-mers containing single-base mismatches were taken, and the circle size distribution was determined as in Fig. 5. ○, No mismatch; ●, G/G mismatch; △, C/T mismatch; □, C/A mismatch.

joints are spaced by 5 or 10 bp along the DNA, a result that was not observed. Finally, the mean circle size for the 32-mer with two flexible joints should be smaller than that for the 32-mer with one flexible joint regardless of the separation of the two joints along the DNA, an expectation that was not fulfilled. Thus the data argue strongly for the presence of a stiff kink very much like those produced by in-phase blocks of 4–6 adenines.

Hare *et al.* (11) and Roy *et al.* (12) presented models based on NMR data showing how single extra bases stacked into the helix could produce a wedge that kinks the helix. Can stacking-in be equated with a kinked DNA, and looping-out with a nonkinked DNA? We feel that this relation *should not be made* based on the data presented here. First, the electrophoretic and EM assays provide no direct measurement of base stacking. Second, to our knowledge, there is no physical data showing that in solution (as contrasted to the crystal form) looped-out bases do not also kink the helix. Finally, were the relation above to hold, then the consistency of the results obtained here would mean that in all cases the bases were stacked in, including molecules containing 4 extra bases at a single site and molecules in which the extra bases were cytosine and guanine as well as adenine. Nonetheless the NMR models for the stacked-in state (11, 12) are certainly consistent with the kinking observed here. Clearly it will be essential in future studies to correlate the intra- or extrahelical nature of an extra base with its effect on kinking the DNA. Possibly this might be done by following the temperature dependence of the electrophoretic retardation of a DNA containing an extra base where from other studies (17) the base could be shown to stack in or not depending on temperature.

Our observation that the linear ligation products of the 32-mers containing single-base mismatches did not show any mobility retardation relative to the 32-mer containing no lesion is not surprising, since other physical studies have failed to detect any marked perturbation in the helix due to the presence of a single-base mismatch (9, 24–26) and Thomas *et al.* (18) observed no gel retardation due to a single-base mismatch in a 268-bp fragment. However, if a mismatch had generated a flexible joint in the DNA, the ligated DNAs might have shown a downward shift in the mean circle size, while not showing any marked retardation in gel mobility. No such shift was observed for the three mismatch-containing 32-mers examined. The methyl-directed repair system in *E. coli* corrects both mismatches and base deletions/insertions (9) even though it appears that deletions strongly kink the helix whereas mismatches do not. On the other hand, thymine dimers, which kink the helix (19), are repaired by a different repair system. A challenge for future studies will be to understand what features of these lesions in DNA dictate which enzyme system is utilized in repair.

When single-stranded RNA or DNA folds to create complex secondary structures, the resulting molecules consist of duplex rods containing perfectly and imperfectly paired bases linked by junctions containing extra bases on one of the opposing strands. From the data presented here, we argue that for DNA, junctions between two duplex segments that contain 1–4 extra bases create stiff kinks that hold the two duplex segments at precise angles relative to each other, the angle depending on the number of extra bases. If these conclusions hold for RNA, then such junctions could play a critical role in determining the three-dimensional architecture of natural RNA.

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