DNA Hybrids Stabilized by Heterologies†

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ABSTRACT: The double D-loop DNA hybrid contains four DNA strands following hybridization of two RecA protein coated complementary single-stranded DNA probes with a homologous region of a double-stranded DNA target. A remarkable feature of the double D-loop DNA hybrids is their kinetic stabilities at internal sites within linear DNA targets after removal of RecA protein from hybrids. We report here that heterologous DNA inserts in one or both probe strands affect the kinetic stability of protein-free double D-loop hybrids. DNA heterologies normally distort DNA−DNA hybrids and consequently accelerate hybrid dissociation. In contrast, heterologous DNA inserts impede dissociation of double D-loops, especially when the insert sequences interact with each other by DNA base pairing. We propose a mechanism for this kinetic stabilization by heterologous DNA inserts based on the hypothesis that the main pathway of dissociation of double D-loop DNA hybrids is a DNA branch migration process involving the rotation of both probe−target duplexes in the hybrids. Heterologous DNA inserts constrain rotation of probe−target duplexes and consequently impede hybrid dissociation. Potential applications of the stabilized double D-loops for gene targeting are discussed.

Sequence-specific targeting of double-stranded DNA by relatively short DNA probes is applicable for gene cloning, mutagenesis, mapping, and regulation (for review, see *1*). One important class of the hybrid DNA products of the targeting reaction are commonly known as DNA displacement loops (D-loops) (Figure 1). The simplest example is a single D-loop formed by hybridization of a single-stranded DNA probe within a homologous region of a double-stranded target DNA. The incoming single-stranded probe forms Watson−Crick base pairs with the complementary strand of the target double-stranded DNA, and the homologous strand is displaced.

Under physiological conditions, formation of D-loops is mediated by RecA protein or its analogues (*2*−*5*). At elevated temperatures with negatively supercoiled target DNA, D-loops form spontaneously (*6*). When a second DNA probe strand, complementary to the first (and consequently complementary to the displaced strand of the target), is added to a single D-loop, a double D-loop DNA hybrid is formed (*7*, *8*). The formation of double D-loop DNA hybrids in RecA-mediated hybridization reactions has been demonstrated in both linear and negatively supercoiled double-stranded DNA targets (*7*, *8*). These double D-loop hybrids are obtained by either simultaneous or sequential addition of RecA protein coated complementary single-stranded DNA probes to the double-stranded DNA target (*7*).

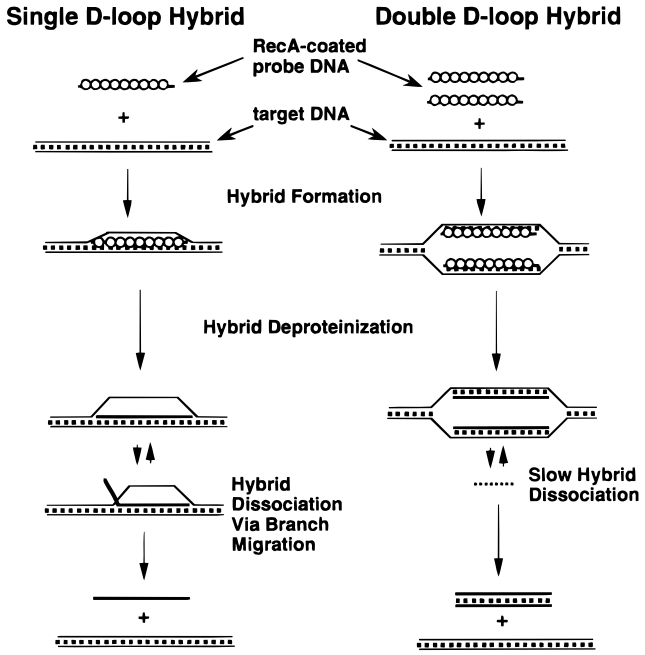

FIGURE 1: Single and double D-loop hybrid formation and dissociation. The branch migration pathway of single D-loop dissociation is established (*9*). Under near-physiological conditions, this process is relatively fast for linear or nicked target DNA, but it is slow for negatively supercoiled target DNA. For double D-loops, dissociation is slow even within linear target DNA. The double D-loop dissociation pathway is unknown, and a possible model is discussed in the present work.

To understand both thermodynamic and kinetic similarities and differences between single and double D-loop hybrids, we examined the dissociation of protein-free D-loop hybrids. With either linear or nicked circular target DNAs, formation of D-loops does not affect the overall target DNA conforma-

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tion. In contrast, within negatively supercoiled target DNAs, D-loops are stabilized by a decrease in free energy due to removal of negative superhelical turns in hybrids (*9*). D-loop hybrid dissociation is driven by a gain in entropy due to separation of the dissociated products. The hybrid dissociation process is reversible if the gain in entropy is compensated by additional DNA base pairing within the dissociating hybrid. In the case of D-loops, the products of dissociation (i.e., intact double-stranded target DNA and single- or double-stranded free probe, in the cases of single or double D-loop, respectively) contain the same total number of base pairs as in the initial D-loop. Thus, dissociation proceeds without decreasing the total number of DNA base pairs. In addition, target DNA duplexes which contain D-loops should have structural distortions in comparison with intact target DNA which provide an additional driving force for dissociation. Consequently, in the case of linear (or nicked) target DNAs, the dissociation of both single and double D-loops is irreversible. However, there is a dramatic difference in the kinetic stabilities (i.e., characteristic times of dissociation) between these DNA structures. For example, the estimated time of dissociation for single D-loops having a length of about 100 bp under near-physiological ionic, pH, and temperature conditions is less than 1 s (*9*). In contrast, the time of dissociation for double D-loop of a similar length under similar conditions is at least several hours (*7*, *8*).

To understand these dramatic differences in kinetic stabilities between single and double D-loops, we examined pathways of dissociation of these two DNA structures (Figure 1). Within a single D-loop, a simple DNA branch-migration process is possible, resulting in one base of the probe DNA in the probe−target duplex substituted by one base of the displaced target DNA strand, and vice versa (*10*). Due to this process, the junction between probe−target and target−target DNA duplexes migrates randomly. Occasionally, it reaches the edge of the position of the probe−target duplex, and then the probe DNA strand irreversibly dissociates from the hybrid. Each step of the branch migration pathway of hybrid dissociation is isoenergetic because a newly formed base pair is equivalent to one formed by a displaced DNA base. The isoenergetic characteristics of DNA branch migration make this pathway of DNA hybrid dissociation much faster than dissociation via denaturation of the probe−target duplex, which requires overcoming a large energy barrier. In the case of double D-loop hybrids, the situation is different. To form one base pair of the target−target duplex, it is necessary to denature two base pairs of probe−target duplexes. Thus, in the first stage, the process of double D-loop hybrid dissociation is not isoenergetic and is significantly shifted toward probe−target duplex re-formation. However, if several DNA bases from the flanks of the probe−target duplexes open occasionally due to thermal fluctuations, then the probe−probe duplex can be nucleated. After nucleation of the probe−probe duplex, the dissociation of the double D-loop hybrid can proceed isoenergetically by migration of a four-way DNA junction formed by target−target, probe−probe, and two probe−target duplexes (see Discussion).

Here we investigated the effect of heterologous DNA inserts in the probe (i.e., inserts which cannot interact with the target DNA) on double D-loop hybrid dissociation. On the one hand, inserts could accelerate double D-loop hybrid dissociation because they distort the probe−target duplex, and thus facilitate its displacement. On the other hand, bulky heterologous inserts could produce an opposite effect by sterically constraining DNA four-way junction migration, especially if they form a complex with each other and "fasten" two probe−target duplexes together. To test which effect of the heterologous inserts predominates, we designed several different DNA probes with heterologous DNA inserts able to interact with each other via Watson−Crick base pairing or guanine quadruplex formation (*11*).

MATERIALS AND METHODS

Oligonucleotide Probes and Target DNA. Figure 2 shows DNAs used in this study. For one pair of Watson−Crick duplex forming heterologous inserts, we chose the $d(GT)_n$/$d(CA)_n$ sequence which has a propensity to form left-handed Z-DNA under topological strain (*12*). This choice of insert sequences is explained under Discussion. Probe oligonucleotides were purchased from the Midland Certified Reagent Co. Oligonucleotides were additionally purified by electrophoresis on 6% denaturing polyacrylamide gels containing 8 M urea. After elution from the gel in TE[1] buffer (10 mM Tris HCl, 1 mM EDTA, pH 8), oligonucleotides were passed through microcentrifuge tube filters (PGC Scientific), gel-filtered through G-25 columns (Pharmacia), precipitated by ethanol, and dissolved in DNA probe−storage buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA) to a final concentration of 1 ng/μL. Radiolabeling of oligonucleotides with [γ-^{32}P]-ATP was performed with T4 polynucleotide kinase (Life Technologies, Gibco BRL). Oligonucleotides were purified on denaturing polyacrylamide gels as described above for unlabeled oligonucleotides except the precipitation step was omitted after the G-25 column.

Targeting Reactions. pBluescript II SK(−) plasmid (Stratagene) was used as the DNA duplex target in all experiments and was purified using a QIAfilter Plasmid Maxi Kit (QIAGEN). Purified plasmid was predominantly negatively supercoiled DNA. The general scheme for forming probe−target hybrids is shown in Figure 3. Complementary oligonucleotides (one of which was ^{32}P-labeled) were coated with RecA protein in separate tubes. During RecA protein coating reactions 21 μL of each oligonucleotide (1 ng/μL) was mixed with 4.2 μL of coating buffer (100 mM Tris−acetate, 500 mM sodium acetate, 20 mM magnesium acetate, 10 mM DTT, 50% glycerol, pH 7.5), 4.2 μL of 20 mM magnesium acetate, and 6.3 μL of 16.2 mM ATPγS (Boehringer Mannheim). Then 7 μL of RecA protein solution [143 ng/μL, obtained from a concentrated RecA protein solution of 3 mg/mL (Boehringer Mannheim) by dilution with RecA-storage buffer (20 mM Tris−acetate, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 50% glycerol)] was added to each oligonucleotide sample. The final concentrations of DNA and RecA in coating mixtures corresponded to 2.4 DNA bases per 1 RecA molecule. Mixtures were incubated at 37−38 °C for 30 min. To initiate the targeting reaction, the target plasmid (4.9 μg) in 70 μL of 18 mM magnesium acetate, 9 mM Tris-HCl (pH 7.5), and 0.09 mM EDTA was added directly to the RecA-coated labeled oligonucleotide. Unlabeled RecA-

[1] Abbreviations: TAE, Tris acetate−EDTA buffer; TE, Tris−EDTA buffer.

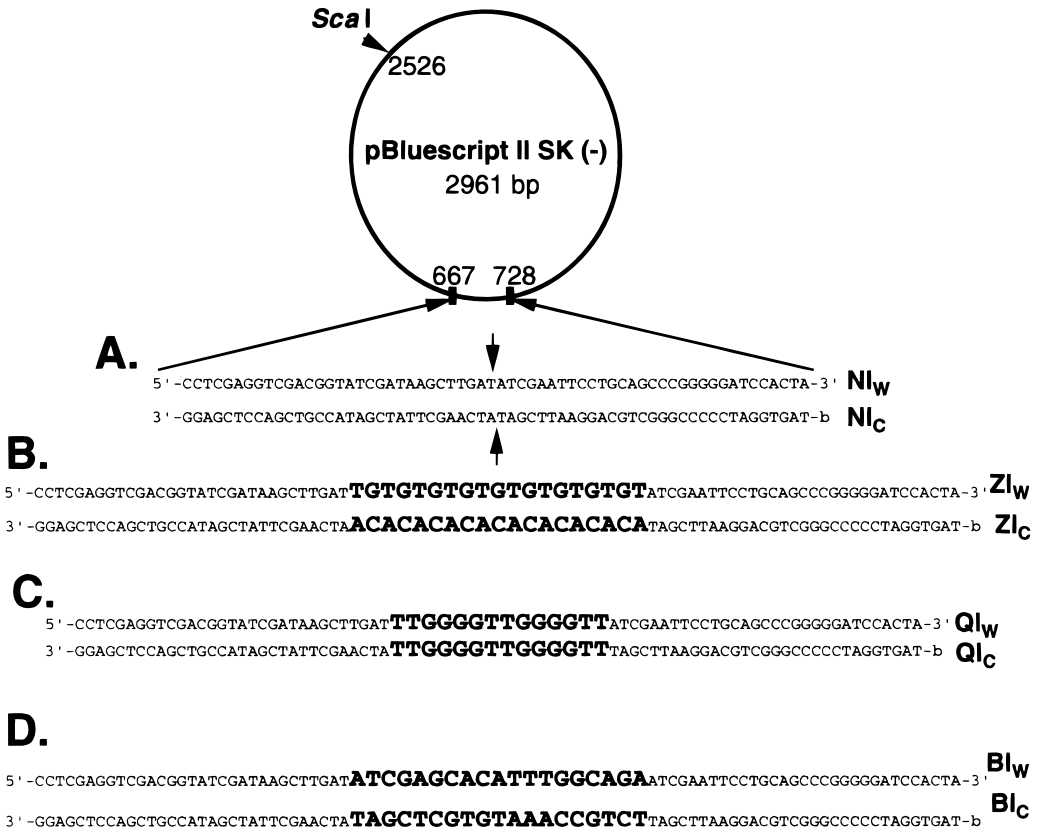

FIGURE 2: DNA probes. (A) Oligonucleotides NI_w and NI_c (No DNA Insert; W, Watson DNA strand; C, Crick DNA strand) are completely homologous to the region of target plasmid pBluescript II SK(−) (from nucleotide positions 667 to 728). (B) Oligonucleotides ZI_w and ZI_c (Z-DNA-forming Insert) differ from NI_w and NI_c by the addition of the heterologous insert sequences $(AC)_9A$ and $(TG)_9T$, respectively, as shown by short arrows. (C) Oligonucleotides QI_w and QI_c (Quadruplex DNA-forming Insert) contain the heterologous insert sequence $T_2G_4T_2G_4T_2$. (D) Oligonucleotides BI_w and BI_c (B-DNA-forming Insert) contain irregular complementary inserts of the same length and GC content as ZI_w and ZI_c; "b", biotin.

coated oligonucleotide was then immediately added to the mixture. Samples were incubated for 1.5 h at 37−38 °C. Next, 16 μL of 10% SDS was added; the mixture was vortexed and incubated for 5 min at room temperature; and 170 μL of phenol−chloroform−isoamyl alcohol (25:24:1) (Life Technologies, Gibco BRL) was added, vortexed, and centrifuged for 1 min. The aqueous fraction containing DNA was removed into a fresh tube, and the same extraction procedure was repeated using chloroform. DNA was precipitated by the addition of 0.1 volume of 3 M NaOAc and 3 volumes of ethanol, air-dried for 10−15 min, and dissolved in 28 μL of DNA probe−storage buffer.

DNA Hybrid Stability. Eleven microliters of DNA hybrid-containing solution was mixed with 25 μL of the probe−storage buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA), 4 μL of 10×REact 6 buffer (Gibco BRL; 1×REact 6 buffer is 50 mM Tris-HCl, 6 mM $MgCl_2$, 50 mM NaCl, 50 mM KCl, pH 7.4), and 3.2 μL of *Sca*I restriction enzyme (Life Technologies, Gibco BRL). Restriction digestion was for 1.5−2 h at 37−38 °C. Next, 57 μL of 1×REact 6 buffer was added to the sample. Aliquots (24 μL) of the resulting mixture were placed in four thin-walled PCR tubes and incubated at 65 °C in a PCR machine with a heated lid to prevent evaporation (PTC-100, MJ Research). All tubes were simultaneously placed in the PCR machine, and the tubes were quickly removed to dry ice at defined time points. Next the samples were thawed and loaded onto a 1% agarose gel/TAE buffer (40 mM Tris−acetate, 1 mM EDTA, pH 8). Electrophoresis was performed at room temperature at ~1 V/cm for 20 h. After electrophoresis, DNA in gels were stained with ethidium bromide (1 μg/mL) in TAE and photographed under UV light with Polaroid film. In addition, the positions of all DNA bands observed under UV light after ethidium bromide staining were marked on transparent film to identify positions of DNA bands on autoradiograms. Next gels were dried on DE-81 anion exchange chromatography paper (Whatman) and either exposed to X-ray film (Kodak) or placed on a phosphor imager screen (Molecular Dynamics). Quantitative analysis of gels was performed using Image Quant software (Molecular Dynamics).

For stability measurements at different ionic conditions, after restriction digestion hybrids were filtered through a G-25 column equilibrated with 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA to remove restriction buffer, and then appropriate buffer was added.

RESULTS

Formation of Double D-Loop DNA Hybrids. The kinetic stabilities were measured for double D-loop hybrids with different heterologous inserts within the linear target DNAs. The length of homology between the probe and the target DNAs was 62 bases. A negatively supercoiled DNA was used as an "intermediate" target because the linear DNA targeting efficiency with short probes is relatively low (*7*,

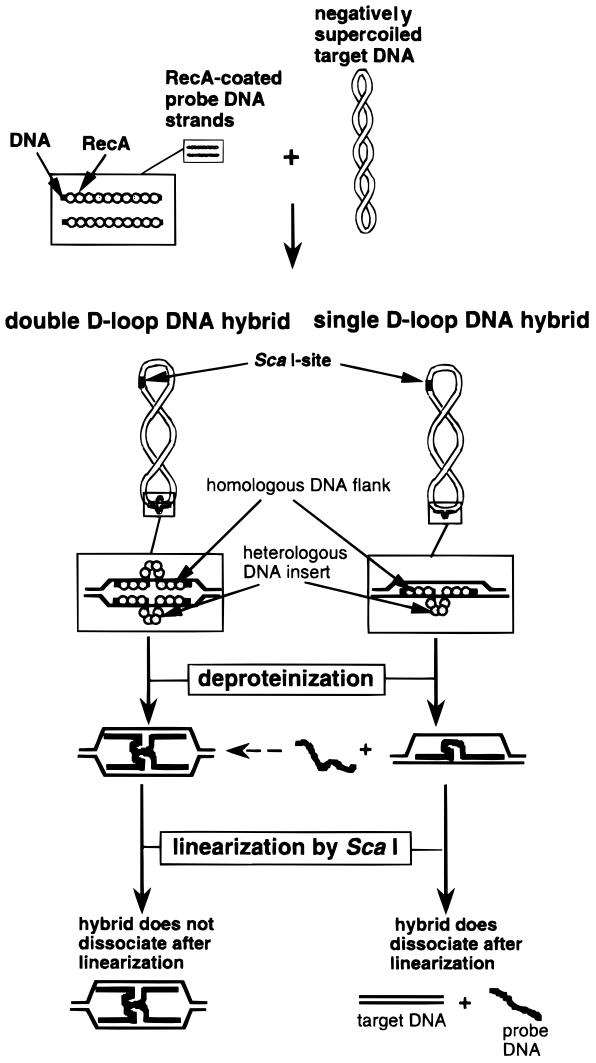

FIGURE 3: Formation of double D-loop DNA hybrids. RecA protein coated complementary single-stranded DNA probes (small circles symbolize RecA protein) hybridize with negatively supercoiled double-stranded DNA targets. Formation of the probe−target hybrids causes partial relaxation of negative superhelical stress in the target DNA. This is schematically shown by a decreased number of plectonemic negative superturns in the target. The heterologous DNA inserted into the probe DNA strand is looped out from the probe−target duplex. Heterologous DNA inserts are completely coated with RecA protein, which can prevent them from interaction with each other before RecA is removed. Both single and double D-loops can be formed by this reaction. Both these hybrid structures survive deproteinization within supercoiled hybrids, but only double D-loops are stable after linearization of the deproteinized hybrid. After linearization, deproteinized single D-loop DNA hybrids rapidly dissociate, producing single-stranded DNA probe and double-stranded DNA target. Dashed arrows indicate that both during and after deproteinization, single D-loop hybrids can be converted to double D-loop hybrids by RecA-independent hybridization between the displaced strand of a single D-loop and the free single-stranded DNA probe.

8). The general strategy of these experiments is shown in Figure 3. First, RecA-coated single-stranded DNA probes were hybridized with negatively supercoiled target DNA producing single and double D-loop hybrids. Hybrids were deproteinized and linearized by *Sca*I restriction digestion at a site away from the region of double D-loop formation (Figures 2 and 3). Both single and double D-loops formed in negatively supercoiled DNA are expected to be stable after deproteinization because they are stabilized by partial

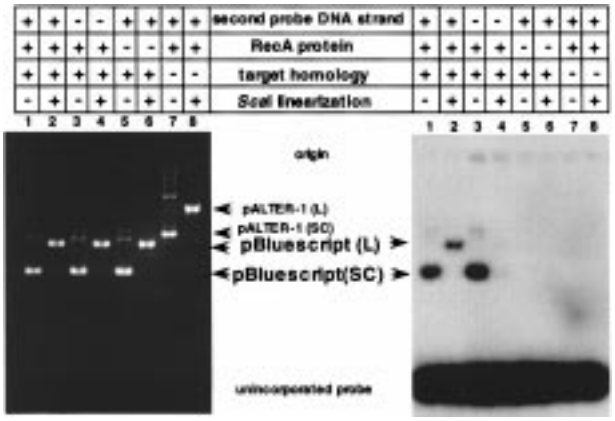

FIGURE 4: Both complementary DNA probe strands are required to stabilize probe−target hybrids after linearization. Two complementary single-stranded DNA probes were targeted to the homologous region of pBluescript II SK(−) plasmid as described under Materials and Methods. As controls for specificity, pALTER-1 (Promega) was used, which does not contain homology with the DNA probes. After the targeting reaction and deproteinization, each sample was separated into two equal parts. To one part was added *Sca*I restriction enzyme (designated as *Sca*I linearization +), and restriction was performed for 2 h at 37−38 °C. Designations of fractions on the gel are as follows: SC, supercoiled DNA; L, linear DNA. The left panel is a photograph of the gel stained with ethidium bromide, and the right panel is an autoradiograph of the same gel. The minor slower migrating DNA bands seen in the photograph in lanes with supercoiled target DNAs are open circular and supercoiled dimers of the target plasmids. Only in the presence of both DNA probe strands (right panel, lane 2) do probe−target hybrids survive linearization.

relaxation of superhelical tension in the target DNA (9). However, only double D-loop hybrids are expected to survive linearization of the target (*7*, *8*). Figure 4 shows DNA hybrids surviving linearization are indeed double D-loops. In these experiments, one of the probe strands was radioactively labeled and monitored by autoradiography (right panel). The position of the target plasmid DNA was monitored by ethidium bromide staining (left panel). The probe comigrated with supercoiled target DNA, showing formation of the probe−target hybrid (Figure 4, right panel, lanes 1, 3). As expected, in the case of supercoiled target DNA, hybrids were observed both in the presence and in the absence of the second probe strand. After linearization of the target plasmid by *Sca*I restriction enzyme, the hybrid comigrating with the linear target DNA was observed only if both probe strands were present (Figure 4, right panel, lane 2). Thus, in linear target DNA, both probe strands are required for hybrid stabilization. This shows linearized hybrids contain double D-loops. Linearized double D-loop hybrids were tested for kinetic stability. Kinetic stabilities of different kinds of hybrids were estimated by the rates of their dissociation at an elevated temperature (65 °C).

Heterologous DNA Inserts Kinetically Stabilize Double D-Loop Hybrids. Figure 5A shows double D-loops formed by four different combinations of completely homologous probe strands (NI_w and NI_c) and probe strands with quadruplex-forming heterologous inserts (QI_w and QI_c). Probe−target hybrids formed by completely homologous probe strands (NI_w/NI_c) (lanes 13−16) were barely detectable after 2 min of incubation at 65 °C (lane 14). In the case of quadruplex-forming probe strands (QI_w/QI_c) (lanes 1−4), the hybrids are observed after 20 min of incubation at 65 °C

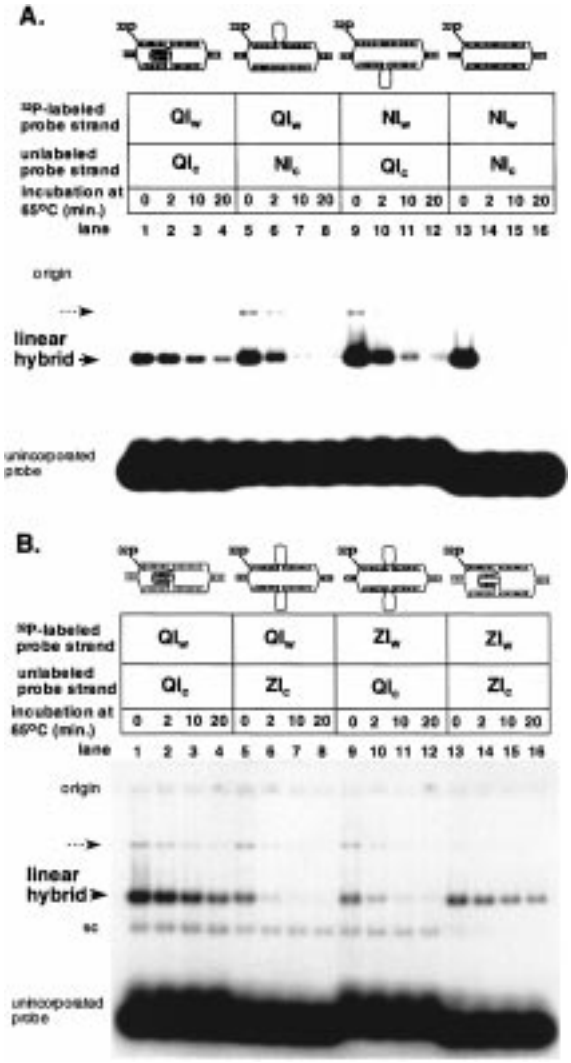

FIGURE 5: Double D-loop hybrid stability. *Sca*I-linearized probe−target hybrids were incubated at 65 °C for different times, and the amounts of the hybrids remaining after incubation for various times were monitored by gel electrophoresis. The types of hybrids formed by different combinations of probe DNA strands are shown at the top. (A) Combinations of completely homologous probe strands (NI, No DNA Insert) and probe strands with Quadruplex-DNA-forming Inserts (QI) are shown. The slowest dissociation of double D-loop hybrids occurred when both strands contain quadruplex-forming DNA insert sequences (lanes 1−4). (B) The same experiment as shown in (A), but with ZI (Z-DNA-forming Insert) probes substituted for NI probes. The "matched" combinations (lanes 1−4 and 13−16) produce hybrids with increased kinetic stability compared to "mixed" combinations (lanes 5−8 and 9−13). This indicates that complex formation between heterologous inserts significantly contributes to double D-loop stabilization. In some cases, in addition to linearized hybrids, small amounts of uncut supercoiled (SC) hybrids remained. In contrast with linear hybrids, the amount of supercoiled hybrid did not change significantly during incubation at 65 °C. This result is expected because supercoiled hybrids are much more stable. The minor DNA fraction shown by the dashed arrow was not detectable by ethidium bromide staining and probably represents hybrids formed with a linear dimer of the target plasmid (i.e., the dimer plasmid molecule appears to be cut only in one *Sca*I site). It also could represent dimeric double D-loop hybrid formed via base pairing between heterologous inserts which belong to different hybrid DNA molecules.

(lane 4). The quantitation by phosphorimaging in this and other similar experiments shows that the half-time of dissociation for the QI_w/QI_c probe is about 5 min. In the case of "mixed" probe strands containing combinations (QI_w/NI_c and NI_w/QI_c) (lanes 5−8 and 9−12, respectively), "intermediate" kinetic stabilities were observed. These measurements show that the portions of these "half-heterologous" double D-loop hybrids which survived after 2 min of incubation are significantly larger (though still less than 50%) than in the case of completely homologous hybrids. These data show stabilization of the double D-loop by heterologous inserts. The increased kinetic stability of the QI_w/QI_c hybrid versus half-heterologous (QI/NI) hybrids suggests that quadruplex formation within the QI_w/QI_c hybrid significantly contributes to kinetic stabilization of the probe−target hybrid. However, it does not exclude the possibility that this increased kinetic stability is caused by steric factors, rather than specific interactions between heterologous DNA inserts. To address this possibility, we performed similar experiments with combinations of probes containing quadruplex-forming heterologous inserts (QI_w and QI_c) and Watson−Crick duplex forming inserts prone to Z-DNA formation (ZI_w and ZI_c). We tested all four possible combinations of probes: QI_w/QI_c, QI_w/ZI_c, ZI_w/QI_c, ZI_w/ZI_c. In the QI_w/QI_c and the ZI_w/ZI_c (matched) combinations, heterologous inserts are able to form stable complexes (quadruplex and Watson−Crick duplex structures, respectively). In the QI_w/ZI_c and the ZI_w/QI_c (mixed) combinations, stable complex formation between heterologous inserts is not expected. If differences in the stabilities of double D-loop hybrids were due to complex formation between heterologous inserts, then "matched" combinations of probes would tend to produce more stable hybrids than "mixed" ones. If the differences in stabilities were due to steric effects, "mixed" combinations should produce double D-loop hybrids of intermediate stability. Figure 5B shows that the hybrids with "matched" combinations of probes (QI_w/QI_c, lanes 1−4; and ZI_w/ZI_c, lanes 13−16) dissociate more slowly (i.e., they are more kinetically stable) than hybrids with "mixed" combinations of probes (QI_w/ZI_c, lanes 5−8; and ZI_w/QI_c, lanes 9−12). Figure 6 includes data for all combinations of QI, ZI, and NI probe strands and the schematics of proposed hybrid structures. The half-time of dissociation for double D-loops formed by "matched" combinations QI_w/QI_c and ZI_w/ZI_c is about 5 and 10 min, respectively, and is less than 2 min for the rest of the combinations. Thus, complex formation between heterologous inserts can significantly contribute to double D-loop hybrid stabilization. However we did not detect an apparent stabilization of double D-looped hybrids formed by the matched combination BI_w/BI_c in which complementary heterologous inserts have the same length and GC content as in ZI, but are not prone to Z-DNA formation, in comparison with the mixed combinations BI_w/ZI_c and ZI_w/BI_c: for all three of these combinations, half-dissociation times of the hybrids were less than 2 min (data not shown) while for ZI_w/ZI_c this time was more than 10 min. This can be explained by topological constraints which appear during interaction between heterologous inserts which can be overcome by Z-, quadruplex-, and triplex-forming sequences, but not by usual B-DNA-forming sequences (see Discussion).

Additional evidence that the G-quartet-containing quadruplex structure is responsible for stabilization of the QI_w/QI_c hybrid is that the stabilization effect is dependent on the type of monovalent cations: when potassium and sodium [which promote G-quartet formation (*11*, *13*)] are replaced by lithium [which does not promote G-quartet formation

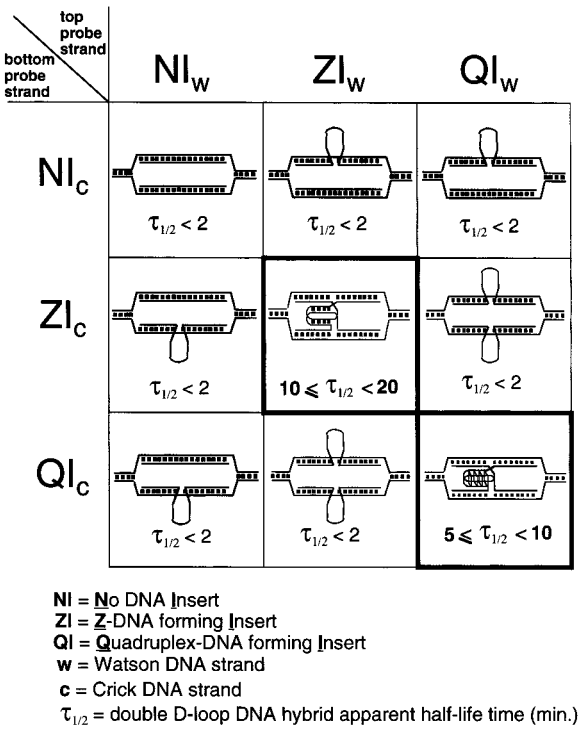

FIGURE 6: Comparison of the rates of dissociation of double D-loop hybrids. The apparent half-lifetime, $t_{1/2}$, is the time of incubation (minutes) in which 50% of the double D-loop hybrids dissociate. The longer the $t_{1/2}$, the greater the kinetic stability of the hybrids. The double D-loop hybrids formed by "matched" combinations of probes QI_w/QI_c and ZI_w/ZI_c have increased kinetic stabilities in comparison with all the other hybrids. Small black squares designate Watson−Crick base pairing, and thin lines designate pairing between guanines in the quadruplex.

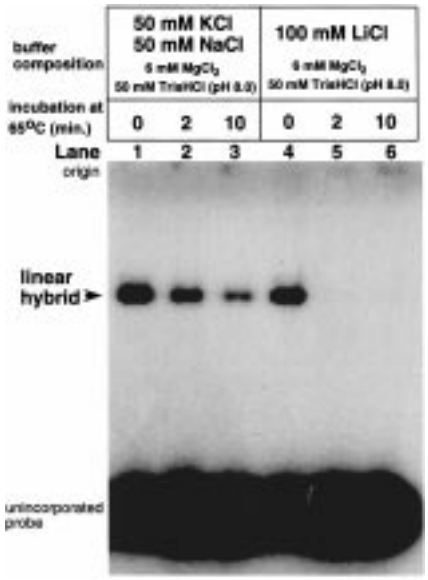

FIGURE 7: Dependence of quadruplex-forming hybrid stability on monovalent cations. In the presence of sodium and potassium ions, significant amounts of hybrids survive after 10 min of incubation at elevated temperature (lane 3), while in the presence of the equivalent amount of lithium ions, hybrids are barely detectable after 2 min of incubation under the same conditions (lane 5).

(*13*)], the stabilizing effect disappears (Figure 7). As expected, for the Z-DNA-forming heterologous insert, this dependence on monovalent cations was not observed (data not shown).

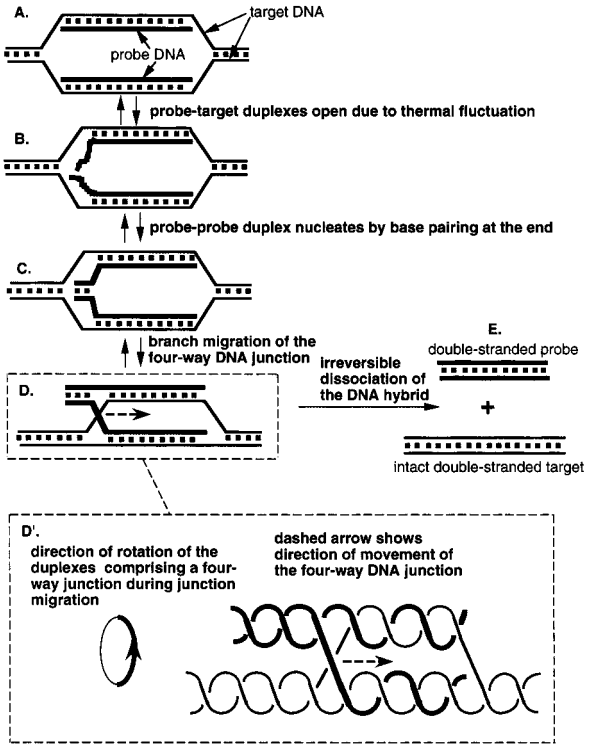

FIGURE 8: Double D-loop DNA hybrid dissociation pathway. (A) Double D-loop DNA hybrid structure with complete pairing between the probe and the target DNA strands. (B) Probe−target DNA duplexes denaturing at the ends due to thermal fluctuations. (C) Nucleation of probe−probe duplex by Watson−Crick base pairing between the ends of the probe DNA strands leads to formation of a DNA four-way junction. (D) DNA four-way junction migrates randomly along the double D-loop hybrid until it occasionally reaches the right edge position, followed by irreversible dissociation of the double D-loop hybrid. (D′) Magnified view of (D) showing the direction of rotation of the DNA duplexes during four-way junction migration. When the four-way junction moves from the left to the right, the probe DNA strands (thick lines) and the target DNA strands (thin lines) spool from probe−target duplexes to probe−probe and target−target duplexes. (E) Products of dissociation of the double D-loop hybrids are intact double-stranded target DNA and double-stranded probe DNA.

DISCUSSION

We observed that heterologous inserts within the probe DNA kinetically stabilize double D-loop hybrids, despite the fact these inserts do not participate in the probe−target interactions and distort the probe−target hybrids. To explain this observation, we propose the following model of double D-loop hybrid dissociation.

Stabilizing Effects of Heterologous DNA Inserts Can Be Explained by a Four-Way Junction Migration Model of Double D-Loop Hybrid Dissociation. Figure 8 shows a model for double D-loop dissociation via DNA four-way junction migration. During the four-way junction migration process, the total number of base pairs does not change (i.e., this process is isoenergetic). The isoenergetic pathway of double D-loop dissociation via migration of four-way junctions appears more probable than dissociation via the energetically unfavorable denaturation of one or both probe−target duplexes. However, the first stage of this pathway, the nucleation of the four-way junction, is preceded by the uncompensated denaturation of several base pairs (Figure 8, from A to C). This creates an energetic barrier for

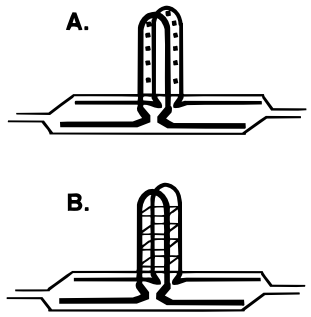

FIGURE 9: Structures of double D-loop hybrids with interacting heterologous inserts. Base pairing is shown only within the complex between heterologous inserts. (A) Heterologous inserts form Watson−Crick base pairs (designated by small black squares). (B) Heterologous DNA inserts form a quadruplex. Thin lines designate base pairing between guanines in the quadruplex.

nucleation, making this process relatively slow. A slow nucleation step can explain the kinetic stability of relatively short (<100 bp) double D-loops. This is in accordance with the fact that the slow initiation step dramatically impedes the duplex displacement via four-way junction branch migration (*14*). It is also possible that the within double D-loops the rate of four-way junction migration is slower than the migration rate for "cruciform-like" systems (*15*) because, within a double D-loop, a four-way junction might be more prone to adopt a "folded" conformation (*16*, *17*) for which the rate of branch migration is slower (*18*).

During four-way junction migration, DNA strands are spooled from one duplex region to the other. This spooling is accompanied by the synchronized rotation of all duplex regions involved in the process (Figure 8D′). Heterologous DNA inserts would constrain the rotation and consequently impede double D-loop hybrid dissociation. This effect would be stronger when both probe DNA strands contain heterologous DNA inserts which can interact with each other, but it also might be detectable in cases when bulky non-interacting DNA inserts create steric obstacles to rotation. We refer to the positive effects of heterologous inserts on double D-loop kinetic stability as "anti-rotational locks".

Our experiments (Figure 5) demonstrate increased kinetic stabilities of double D-loops formed by probes with heterologous inserts as compared to double D-loops formed by completely homologous probes. These effects are more pronounced in cases of interacting heterologous inserts. These observations are in agreement with the anti-rotational lock hypothesis. The stabilizing effect of a non-interacting heterologous insert is also apparent in certain cases (Figure 5A). In our experiments, we did not observe apparent increases in stabilities of double D-loops with two non-interacting heterologous inserts versus one (i.e., QI/ZI versus QI/NI probe). This is also consistent with our model, because two non-interacting heterologous inserts would rotate in the same direction during branch migration, so they would not interfere with each other. Comparisons of the effects of non-interacting heterologous inserts for different probe combinations require measurements at lower temperatures, when dissociation of hybrids is slower.

Structures of Complexes between Heterologous DNA Inserts. Predicted structures of the complexes formed between heterologous inserts are shown in Figure 9. Pairing between the homologous flanks of probe DNA strands and the target prevents an intertwining of heterologous inserts within the complex. Thus, within the complex, heterologous inserts from different probe strands must be topologically unlinked (i.e., heterologous inserts form a paranemic joint). This requirement is satisfied in the case of the quadruplex complexes, which result from interaction between two self-folded hairpins formed by each of the heterologous inserts (*11*). In the case of Watson−Crick interactions between heterologous DNA inserts, this requirement is satisfied if the complex contains the same number of right- and left-handed helical turns. This DNA structure could be formed by $d(GT)_n$/$d(CA)_n$ inserts (probe ZI), since this sequence, in addition to a right-handed B-conformation, can also adopt a left-handed Z-conformation (*12*). Watson−Crick base pairing between complementary heterologous inserts within a topologically unlinked complex might also be possible without Z-DNA formation, if the DNA region participating in the base pairing is less than one helical turn, or if left-handed turns are formed by DNA strands intertwining without base pairing. However, in the case of DNA sequences with strong propensities to adopt Z-conformations, the complexes would be much more energetically favorable. The absence of additional stabilization due to the interaction between complementary non-Z-forming inserts in the BI_w/BI_c hybrid is in agreement with this model. However, it is possible that for longer complementary non-Z-DNA-forming heterologous inserts the stabilizing effect can be detected.

H-DNA-like triplexes (for review, see ref *19*) are also examples of DNA structures which are topologically unlinked and consequently might have superior abilities to form "lock" complexes. [Formation of a triplex "lock" within a double D-loop would be similar to "sticky" DNA formation (*20*).] We observed that the heterologous insert previously shown by Dayn et al. (*21*) to adopt a H-DNA-like triplex structure causes strong kinetic stabilization of double D-loop DNA hybrids (data not shown). However, since the same insert is also able to adopt a Watson−Crick duplex structure, estimation of the contribution of triplex formation to double D-loop stabilization requires additional study.

Possible Application of "Locked" Double D-Loops. Locked double D-loops could be used for blocking DNA copying (i.e., transcription and replication) either via structural distortions of copying enzyme recognition sites or by constraining copying enzyme movement along the target DNA. These structural distortions and copying inhibition could also induce mutations in the target DNA, as was shown for DNA triplexes (*22*).

It was demonstrated that double D-loops can be used for affinity capture of specific target DNA (*7*). Greater kinetic stability of locked double D-loops could be useful for DNA hybrid affinity capture, for example, for screening DNA libraries (*23*, *24*).

Complementary single-stranded DNA probes complexed with RecA protein can be used to insert heterologous DNA sequences into DNA targets (*25*). The double D-loop hybrids with heterologous inserts could be recombination intermediates in this process. It will be interesting to correlate stabilities and recombinogenicities of double D-loops with different heterologous DNA inserts.

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