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Sticky DNA, a Long GAA·GAA·TTC Triplex That Is Formed Intramolecularly, in the Sequence of Intron 1 of the Frataxin Gene*

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Friedreich's ataxia is caused by the massive expansion of GAA·TTC repeats in intron 1 of the frataxin (*X25*) gene. Our prior investigations showed that long GAA·TTC repeats formed very stable triplex structures which caused two repeat tracts to adhere to each other (sticky DNA). This process was dependent on negative supercoiling and the presence of divalent metal ions. Herein, we have investigated the formation of sticky DNA from plasmid monomers and dimers; sticky DNA is formed only when two tracts of sufficiently long $(GAA·TTC)_n$ (n = 59–270) are present in a single plasmid DNA and are in the direct repeat orientation. If the inserts are in the indirect (inverted) repeat orientation, no sticky DNA was observed. Furthermore, kinetic studies support the intramolecular nature of sticky DNA formation. Electron microscopy investigations also provide strong data for sticky DNA as a single long triplex. Hence, these results give new insights into our understanding of the capacity of sticky DNA to inhibit transcription and thereby reduce the level of frataxin protein as related to the etiology of Friedreich's ataxia.

Friedreich's ataxia (FRDA)[1] is the most common hereditary ataxia. The gene for this autosomal recessive neurodegenerative disease has been mapped to chromosome 9q13-q21.1 by linkage studies (1, 2). The clinical features and molecular biology FRDA have been reviewed (3–10). The FRDA gene, *X25*, contains seven exons and encodes a 210-amino acid protein called frataxin (8). The vast majority of FRDA patients have an expanded GAA·TTC repeat in the first intron of the frataxin gene. Normal alleles have 6–34 repeats of GAA·TTC repeats, but FRDA-associated alleles have 66–1,700 or more GAA·TTC repeats (3, 8, 10). FRDA is the only triplet repeat disease that has a recessive inheritance pattern and is caused by the expansion of a GAA·TTC repeat (9).

The age of onset and the severity of the disease are correlated with the length of the GAA·TTC repeats (8, 11, 12). Patients carrying expanded GAA·TTC repeats in both alleles have reduced levels of frataxin, and an inverse correlation exists between the size of the GAA·TTC repeat and amount of the frataxin protein (13, 14). This reduction in the amount of the frataxin protein is caused by a diminution in the amount of the frataxin mRNA (8, 15, 16). The amount of the *X25* mRNA is inversely related to the length of the GAA·TTC repeat both *in vitro* (17) and *in vivo* (18). Virtually all workers in the field have proposed that the long GAA·TTC repeats form triplexes (three stranded DNA structures) that are involved in the transcriptional inhibition (16–22). In fact, recent investigations (22) directly demonstrated that sticky DNA (a complex triplex formed at GAA·TTC repeats; described below) inhibits transcription *in vitro*, and *in vivo* investigations (18, 23) are consistent with these results.

Substantial prior investigations (19, 24–29) showed that shorter GAA·TTC inserts (16–58 repeats) adopt triplex structures. DNA triplexes are formed at polypurine·polypyrimidine (R·Y) tracts (for review, see Refs. 30–33). Intramolecular triplexes are formed in negatively supercoiled plasmids and may either be of the R·R·Y or the Y·R·Y type. R·R·Y triplexes are stabilized by divalent metal ions (30–33). Furthermore, intermolecular triplexes have been widely investigated (for review, see Refs. 32 and 33) as formed by the Hoogsteen pairing of synthetic oligonucleotides to duplex tracts containing the R·Y motif. Both intermolecular and intramolecular triplexes are known to inhibit transcription (for review, see Ref. 34).

During the characterization of recombinant plasmids containing very long tracts of GAA·TTC, a novel DNA conformation, sticky DNA, was described for lengths of the TRS found in intron 1 of the frataxin gene of FRDA patients (20). Sticky DNA was stabilized by negative supercoiling as well as divalent metal ions and was characterized as an X-shaped structure in the linearized plasmid molecules. The crossover points of the X-shaped molecules were rigorously shown to occur at the TRS. We hypothesized (20) that individual intramolecular triplexes were formed in separate plasmids that then associated with each other at the triplex regions.

Here we have conducted a further evaluation of the molecular nature of sticky DNA. These results led us to conclude that sticky DNA is only formed intramolecularly from two tracts of GAA·TTC in a single molecule and exists as a single long R·R·Y

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[1] The abbreviations used are: FRDA, Friedreich's ataxia; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; EM, electron microscopy; R, purine; RB, retarded band; σ, superhelical density; TRS, trinucleotide repeat sequence(s); Y, pyrimidine.

triplex. Thus, the previous hypothesis of a strand-exchanged bitriplex model (20) has been modified.

The companion article (35) explores the sequence requirements for sticky DNA formation.

EXPERIMENTAL PROCEDURES

Preparation of Plasmids—Plasmids containing a single GAA·TTC tract (pSPL3 derivatives) which were used in these experiments are shown in Fig. 1 and have been described previously (20, 22). pRW4204 has also been described previously (21). Plasmids with two $(GAA{\cdot}TTC)_{60}$ tracts, which are pBR322 derivatives, are shown in Figs. 4 and 7*A*; construction of these DNAs is described below.

Supercoiled plasmids were purified either by the large scale plasmid isolation method followed by equilibrium centrifugation in a CsCl/ethidium bromide (EtBr) gradient (36) or by the Promega Wizard Plus Minipreps DNA Purification procedure (Promega Corp.) according to the manufacturer's recommendations. Large scale plasmid isolations were performed from 1 liter of overnight cultures of the *Escherichia coli* SURE strain. *E. coli* were transformed using the $CaCl_2$ transformation method (36) and grown in LB medium containing 100 μg/ml ampicillin. After DNA isolation by alkali lysis, plasmids were purified by equilibrium CsCl/EtBr gradient ultracentrifugation overnight. The ultracentrifugation was followed by exhaustive dialysis against three changes of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (TE) buffer.

To purify DNA monomers and dimers, 1–10 μg of the isolated plasmids, which contained monomers, dimers, and higher oligomers in 60 μl of TE buffer containing 10% sucrose and 0.01% bromphenol blue (loading buffer), was loaded onto a 1% agarose gel. Electrophoresis was conducted in 40 mM Tris acetate (pH 8.3), 1 mM EDTA (TAE) buffer at 5 V/cm until the bromphenol blue reached a distance of 6 cm. Gels were stained by EtBr as described elsewhere (36). To prevent DNA damage during UV irradiation, aluminum foil was placed under the gel as a screen while excising the band. The bands corresponding to the monomeric and dimeric isoforms of the plasmids were excised from the agarose gels. DNAs were eluted from the gels using a QIAquick gel extraction kit (Qiagen Inc.) according to the manufacturer's recommendations.

To obtain analytical amounts of the monomeric and dimeric plasmid isoforms, we transformed *E. coli* HB101 with monomers and dimers. Individual colonies were inoculated into 10 ml of LB containing 100 μg/ml ampicillin. After overnight growth, DNAs were isolated by the Promega Wizard Plus Minipreps DNA Purification procedure and analyzed on 1% agarose gels.

When transformations were performed with the purified monomers, electrophoretic analyses of the isolated DNAs revealed that monomers represented more than 90% of the total DNA on a molar basis. When transformations were performed with purified dimers, no monomers were observed.

Cloning a Pair of $(GAA{\cdot}TTC)_{60}$ Tracts into pBR322—A fragment containing the $(GAA{\cdot}TTC)_{60}$ tract was prepared from pRW3804 (18) by *Bss*HII and *Hae*III digestion (New England Biolabs, Inc.) followed by filling in the recessed *Bss*HII 3′ terminus with 0.1 unit of the Klenow fragment of *E. coli* DNA polymerase I (U. S. Biochemical Corp.) and dCTP plus dGTP (0.1 mM each). The sequence of the insert is $GAGGA(GAA)_{50}GAAAAAGAAAA(GAA)_{10}$, which is named $(GAA{\cdot}TTC)_{60}$ for simplicity. The repeating GAA·TTC tract is flanked by 34 bp and 54 bp of the human FRDA gene (8). This blunt ended DNA fragment was used for the cloning of all four plasmids containing the TRS tracts in both orientations relative to the unidirectional ColE1 origin of replication shown in Fig. 4. The digested DNA was electrophoresed in a 7% polyacrylamide gel, stained with EtBr, and the band containing the triplet repeat fragment was excised. The DNA was eluted from the excised band, purified by phenol-chloroform extraction, and precipitated with ethanol (36). The vector was prepared by digesting pBR322 with *Eco*RI and *Hin*dIII followed by filling in the recessed 3′ termini. The ligation and all subsequent cloning steps were performed as described earlier (37). All plasmids were fully characterized by restriction mapping (to determine the orientation and length of the cloned TRS) and dideoxy sequencing of both strands with Thermo Sequenase Radiolabeled Terminator Cycle Sequencing kit (U. S. Biochemical Corp.). This strategy enabled the construction of a set of plasmids (pRW4882–pRW4885) harboring two homologous $(GAA{\cdot}TTC)_{60}$ tracts oriented as direct repeats or inverted repeats (see Fig. 4).

Detection of RB Formation—The presence of sticky DNA in a DNA preparation is determined routinely by the detection of a substantially retarded band (RB) on agarose gel electrophoresis after plasmid linearization. Detection of RB formation was performed as described (20) with some modifications. Cleavage of plasmid DNAs by appropriate restriction enzymes was conducted in NEBuffer 1 (New England Biolabs, Inc.) (10 mM bis-Tris propane HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (pH 7.0 at 25 °C)) supplemented with 100 μg/ml bovine serum albumin if recommended by the manufacturer. Five units of the restriction enzyme/0.5 μg of DNA was used in a total reaction volume of 12 μl. Incubation times at 37 °C were 200 min or overnight; no differences were detected. Chilling to room temperature and addition of the loading buffer, as described above, stopped the reactions.

To identify RB, we incubated the DNA for 10 min at 80 °C in 50 mM EDTA as described (20). RB is converted completely to the linear monomeric form after this treatment. Thus, the heat sensitivity of RB in the absence of divalent metal ions allows us to distinguish between RB and other possible structures.

In some cases, the DNAs were ^{32}P labeled using the *E. coli* DNA polymerase I Klenow fragment end labeling procedure (36) before loading onto the gel.

Electrophoretic separations were conducted in 0.7% agarose gel for pSPL3 derivatives and 1% gel for pBR322 derivatives in TAE buffer at 5 V/cm with a distance of bromphenol blue migration of at least 6 cm. Staining of the gels by EtBr was performed as described elsewhere (36). For the electrophoresis of end-labeled samples, the agarose gels were dried at 65 °C overnight and exposed to Hyperfilm MP (Amersham Biosciences). To quantitate the EtBr-stained gels as well as the autoradiographs, we usually used the Gel Doc 2000 system from Quantity One software (Bio-Rad) for Windows 98. The standard error for RB formation (see Tables I and II) is ±10%.

Are Biological Dimers in the Head-to-tail Orientation or Catenanes?—Two types of determinations were conducted to evaluate whether the biological dimers were two monomer units oriented in the head-to-tail (or tail-to-head) orientation *versus* two interlocked circles or catenanes. First, *E. coli* HB101 was transformed by the $CaCl_2$ method with gel-purified dimers of pRW3822. Ten individual colonies were inoculated into 10 ml of LB medium containing 100 μl/ml ampicillin. After overnight growth, DNA was isolated by the Promega Wizard Plus Minipreps DNA Purification procedure and electrophoresed through 1% agarose gels. Only dimers, no monomers, were observed. Because *E. coli* harbors an active resolvase, monomers should have been observed if the DNA structures were catenated but would not be found in the case of head-to-tail (or tail-to-head) dimers.

In a second approach, UV irradiation experiments were performed to evaluate the presence of catenanes. Gel-isolated supercoiled dimers of pRW3822 were nicked to the extent of complete linearization by UV light (for review, see Ref. 38). If the dimeric structure was a catenane, a significant fraction of the product should be linear monomers after complete linearization. Supercoiled, gel-isolated dimers of pRW3822 (2 μg) in 0.2 ml of TE buffer in a 1-mm width quartz cuvette was treated at room temperature with 254 nm of UV irradiation inside a Bio-Rad GC Gene Linker UV Chamber for 16–1577 s. Samples were removed at seven different time intervals and incubated for 10 min at 70 °C to anneal the sticky ends. All aliquots were then analyzed by 0.7% agarose gel electrophoresis. After staining the gel with EtBr, we observed that the DNA was completely linearized, and the product was the linear dimeric form; no linear monomers were found.

Thus, no catenanes or self-associated monomers were present in the dimeric sample of pRW3822. Hence, we conclude that only head-to-tail (or tail-to-head) biological dimers were present. Of course, this conclusion is warranted only if the sticky DNA is not uniquely cross-linked by UV irradiation under our reaction conditions.

Preparation of pRW3822 Topoisomers—To determine the potential influence of negative supercoiling on the length of the DNA/DNA interacting region, samples of relaxed, native, and negatively overwound pRW3822 were prepared. To obtain completely relaxed samples, 4 μg of the mixture (1:1, w/w) of the supercoiled dimer of pRW3822 and monomer of pUC19 (as a reporter of the values of the negative superhelical density (σ)) was treated with chicken erythrocyte topoisomerase I (prepared by J. E. Larson, this laboratory) in J-1 buffer (10 mM Tris-HCl (pH 7.6), 50 mM KCl, 1 mM EDTA) to relax DNA in the absence of divalent metal ions, or calf thymus topoisomerase I (Invitrogen) in 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 30 μg/ml bovine serum albumin to relax DNA in the presence of divalent metal ions. Incubation of the reaction mixture at 37 °C for 200 min relaxed the DNA completely, to average $-\sigma = 0$. Increasing the incubation time up to 600 min had no detectable influence on the σ.

To obtain the samples of negatively overwound DNA, 4 μg of the mixture (1:1, w/w) of supercoiled dimer of pRW3822 and monomer of pUC19 (as a reporter) were treated with *E. coli* DNA gyrase (a gift from N. P. Higgins) in 35 mM Tris-HCl (pH 7.6), 18 mM potassium phosphate

(pH 7.6), 6.7 mM $MgCl_2$, 5 mM dithiothreitol, 50 μg/ml bovine serum albumin, and 1.5 mM ATP. Incubation of the reaction mixture at 30 °C for 200 min added an additional $-\sigma = 0.025 \pm 10\%$ to the DNA. Increasing the incubation time up to 600 min had no detectable influence on σ. Considering that the density for a natural, isolated plasmid DNA is $-\sigma = 0.05 \pm 10\%$ (39–41), we estimate that the final density is $-\sigma = 0.075 \pm 10\%$.

To determine the distribution of topoisomers, the changes in σ, and to prove that the DNA was relaxed and not nicked, electrophoresis was conducted in the absence or presence of 5 μM chloroquine using pUC19 as a reporter of the σ, as described (42).

Electron Microscopy (EM)—Kleinschmidt's method was used for the preparation of DNA for EM (43). The DNA (100 ng) was added to the hyperphase containing 0.5 M ammonium acetate and 60 μg/ml cytochrome *c*. The DNA was spread immediately onto a hypophase of 0.25 M ammonium acetate. The DNA was adsorbed onto Parlodion-coated grids, stained with uranyl acetate (Ladd Research Industries, Inc.), and rotary shadow cast with Pt:Pd (80:20). After shadow casting, the grids were then briefly coated with carbon. Micrographs were taken on a Phillips CM12 electron microscope at 40 kV. End-to-end lengths as well as the contour lengths were measured using a Summagraphics digitizing tablet coupled to a Macintosh computer programmed with software developed by J. D. G.

To determine the degree of bending within the DNA/DNA interacting regions of the dimeric associated DNAs, DNA was adsorbed onto thin carbon foils, washed, air-dried, and rotary shadowcast with tungsten at high vacuum as described (44). Samples were examined, the curvilinear and straight distance determined as described above, and the degree of bending was determined using the method employed by Wang *et al.* (45).

RESULTS

Requirement for Second GAA·TTC Tract in Same Molecule for RB Formation—Previous studies on sticky DNA were conducted with a mixture of the monomeric, dimeric, and higher oligomeric forms of recombinant plasmids containing a GAA·TTC tract (20–22); the relative ratio of the dimers compared with monomers on a molar basis varied between 0.5 and 2.0 as a function of the TRS length.[2] These mixtures of plasmid oligomeric forms are observed upon isolation of plasmids from recombination-proficient *E. coli* (*e.g.* SURE strain) for plasmids containing the ColE1 origin of replication. Because of the characteristics of the formation of the RB in previous studies (20), we wished to evaluate the formation of RB from purified dimeric or monomeric forms of pRW3822; these topological forms were purified after agarose gel electrophoresis of pRW3822 DNA grown in *E. coli* SURE (see "Experimental Procedures"). The plasmids used in this study are shown in Fig. 1. After purification of the monomeric and the dimeric forms (Fig. 2*A*), the DNAs were cleaved with *Xmn*I or *Eco*RI to reveal RB formation (for detection of sticky DNA, see "Experimental Procedures"). Electrophoretic analyses of the restriction products on an agarose gel (Fig. 2, *A–C*) demonstrates that RB was formed only from the dimeric, not the monomeric form, of pRW3822. Hence, these data suggest that at least two long GAA·TTC tracts must be present in the plasmid to observe sticky DNA. It may be noted that dimeric forms of plasmids isolated from *E. coli* are oriented in the head-to-tail orientation (46).

Studies were also conducted with the purified higher oligomeric forms (*e.g.* trimers, tetramers; see "Experimental Procedures"); as expected, RB was formed in parallel experiments (data not shown).

We also wished to evaluate the necessity of genetic recombination in the formation of sticky DNA. Accordingly, we retransformed *E. coli* RR1 and its isogenic *recA*$^-$ strain HB101 with the monomeric and the dimeric forms of pRW3822. Fig. 2*D* shows that the dimeric form of pRW3822 after harvesting from HB101 reveals sticky DNA after cleavage by *Xmn*I, whereas this structure is absent when the monomeric form is linearized. No differences were found in the ability to form RB for gel-purified monomers and dimers when grown in *E. coli* RR1 (data not shown), HB101, or SURE. Thus, the formation of RB is independent of the presence of an active RecA system.

Effect of Dilution—If the formation of RB is via an intramolecular interaction between the two GAA·TTC tracts within the biological dimers, little effect of dilution of the DNA substrate would be expected. Alternatively, if the formation of RB (sticky DNA) is the result of the interaction of two independent DNA molecules in the solution (bimolecular reaction) (20), a dramatic effect of dilution of the DNA substrate would be expected; the yield of RB should decrease with the square of the concentration of the plasmid DNA. Thus, after a 300-fold dilution of DNA, a 90,000-fold decrease (300×300) would be expected in the formation of RB if the reaction was an intermolecular process.

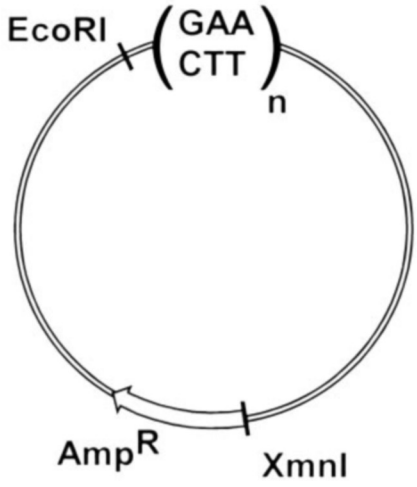
Table I shows the influence of the concentration of purified dimeric pRW3822 DNA on the yield of RB after *Eco*RI cleavage and analysis by electrophoresis on an agarose gel. The purified dimeric pRW3822 DNA was serially diluted. To avoid *Eco*RI star activity (47), the *Eco*RI underwent the same serial dilution procedure; the diluted enzyme was then added to the diluted DNA, and the conditions of the *Eco*RI cleavage were as described under "Experimental Procedures." The maximum dilution of the purified dimers of pRW3822 was 300-fold. Table I shows that dilution of the substrate had little effect on the yield of sticky DNA. Accordingly, these data provide kinetic evidence of the presence of an intramolecular reaction between the two long GAA·TTC tracts within one molecule, as shown in schematic form in Fig. 3. The data shown in Table I are consistent with our previous results (20) on the effect of DNA concentration on RB formation.

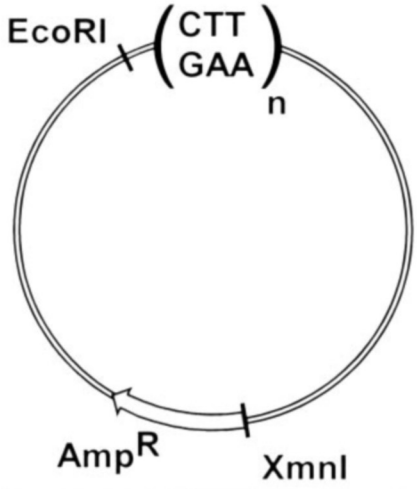
Effect of Length, Orientation, and Sequence of TRS on Sticky DNA Formation—The effect of length and orientation of the GAA·TTC inserts was evaluated with the first five plasmids listed in Table II. The inserts contain lengths of GAA·TTC repeats ranging from 33 to 190 and were in both orientations. Dimers were purified after propagation in *E. coli* SURE strain and were cleaved with *Eco*RI to evaluate the formation of sticky DNA. The data in Table II show that a similar yield of RB was found as discovered previously with mixtures of the plasmid oligomers (20, 22) after correction (subtraction) for the amount of monomer in the final mixture. Parallel studies with the isolated monomeric forms of the plasmids in Table II revealed that no RB was found in any case, as expected. Hence, it is necessary to subtract the percentages of monomers from the total amount of plasmid DNA when evaluating the formation of RB because only the dimeric form has the capacity to generate sticky DNA (Fig. 2).

Table II also shows that modest amounts of substitution of the GGA·TCC repeat within the GAA·TTC repeats will enable the tracts to form RB from the isolated dimeric, but not monomeric, forms. Again, these data are consistent with previous results (21, 22) of the partially substituted inserts. However, no RB was found for the repeating hexanucleotide sequence GAAGGA·TCCTTC as expected from previous studies (20–22) of the mixture of plasmid monomers, dimers, and higher oligomeric forms.

All of the dimeric forms of the plasmids (Table II) are true head-to-tail dimers, not catenanes. This conclusion was derived by nicking the supercoiled dimeric form of pRW3822 to the linear form by UV irradiation as well as by retransforming the dimers into RecA$^-$ *E. coli* HB101 and investigating single colonies (see "Experimental Procedures"). In both types of experiment, if the "dimeric" forms were catenanes, the isolated prod-

[2] A. A. Vetcher and R. D. Wells, manuscript in preparation.





Plasmid	Length of $(GAA \cdot TTC)_n$	Percent of (GGA • TCC) Substitution	Length (bp)
pMP142	33	0	6749
pRW3821	59	0	6827
pMP178	75	0	6745
pRW3822	150	0	7100
pRW3823	270	0	7460
pMP193	130	50	7040
pRW2113	131	20	7043
pRW4222	136	11	7058
pRW3828	190	0	7200
pRW3827	230	0	7340

FIG. 1. **Schematic diagram of plasmids containing a single GAA·TTC tract used in this study.** All plasmids are derivatives of pSPL3 and have been described previously (see "Experimental Procedures"). The orientation of the inserts has also been defined previously (18).

uct should be monomeric DNA. Because no significant amount of monomers was found, we conclude that the biological dimers are, in fact, head-to-tail dimers that are twice the length of the monomeric units. Also, no catenanes were observed by EM.

Dependence of Sticky DNA Formation on the Orientation of Inserts—For the studies with biological dimers (Fig. 2), both GAA·TTC inserts are present in the head-to-tail orientation (46). Thus, focusing on only one of the strands of the duplex, each of the GAA repeat tracts is on the same DNA strand and is in the same $5' \rightarrow 3'$ orientation. To investigate further the effect of TRS orientation on the formation of sticky DNA, four plasmids (Fig. 4) were constructed with the pBR322 vector in all possible orientations of the inserts. For the eight inserts in the four plasmids in Fig. 4, the repeats were ~60 units in length (see "Experimental Procedures").

These plasmids were analyzed for their capacity to form sticky DNA (see "Experimental Procedures"). Each of the plasmids was cleaved by *Acc*I, which has two recognition sites that are separated by the GAA·TTC repeats. Fig. 5 shows that only pRW4882 and pRW4884, which harbor two $(GAA \cdot TTC)_{60}$ tracts in the head-to-tail orientation, exhibited RB formation; no RB was found when the TRS inserts were in the head-to-head or tail-to-tail orientations (indirect repeat orientations). It should be noted that the head-to-tail orientation where RB was observed is the same as for the biological dimers (Fig. 2). Identical results were observed for two derivatives of pSPL3 which harbored $(GAA \cdot TTC)_{150}$ tracts; when the TRS were in the direct orientation, RB was observed, but no RB was found when the tracts were in the indirect orientation. Likewise, plasmids were constructed containing $(GAA \cdot TTC)_{176}$ in pBR322 (35); RB was observed only when the TRS were in the direct repeat orientation but not in the indirect repeat relationship. Thus, the formation of RB was observed for at least two vectors and for three different lengths of GAA·TTC.

These results on the effect of TRS orientation on RB formation are critical to our comprehension of the structure of sticky DNA. We proposed previously (20) that sticky DNA was formed by the intermolecular association of two triplexes between two different plasmid molecules. An alternative hypothesis is that sticky DNA is one long triplex; from the results described herein, we know that sticky DNA is formed only by an intramolecular interaction between a pair of GAA·TTC regions. Thus,

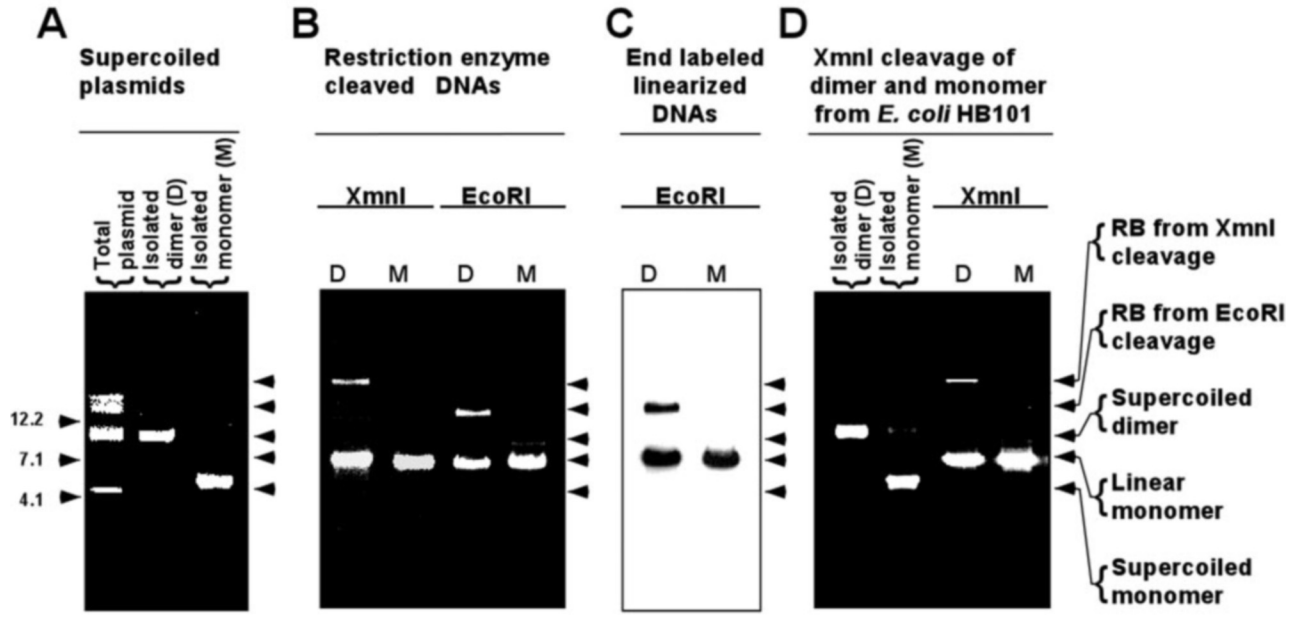


FIG. 2. **Contribution of the dimer or monomer states of pRW3822 to the yield of RB.** *A*, agarose gel (0.7%) analysis of purified supercoiled dimers (*D*) and monomers (*M*) of pRW3822. pRW3822 was isolated (see "Experimental Procedures") from the *E. coli* SURE strain. *B*, analysis of RB formation after restriction enzyme cleavage of pRW3822 in the dimeric or monomeric forms. The dimeric or monomeric forms (*A*) of pRW3822 were cleaved with *Xmn*I or *Eco*RI and analyzed on a 0.7% agarose gel. *C*, analysis of end-labeled *Eco*RI-linearized pRW3822; the *right half* of the gel in *B* was exposed to x-ray film and quantitated (see "Experimental Procedures"). This analysis enabled the determination of the amount of RB formed from the monomeric and dimeric forms of pRW3822. *D*, determination of RB formation from the purified dimeric or monomeric pRW3822 forms analyzed after *Xmn*I cleavage. The plasmid was isolated from *E. coli* HB101 which is RecA$^-$. The analyses, performed in a 0.7% agarose gel, were as described above.

TABLE I
Independence of yield of RB on pRW3822 concentration

To determine the influence of the $(GAA{\cdot}TTC)_{150}$ concentration on the yield of RB after *Eco*RI cleavage, we performed the cleavage step after serial dilution of pRW3822 DNA. To avoid star activity, *Eco*RI underwent the same procedure of serial dilution. Then the corresponding solutions were mixed together. All other conditions of *Eco*RI cleavage were as described under "Experimental Procedures." The maximum dilution of pRW3822 was 300-fold.

pRW3822 concentration	Yield of RB
μg/ml	%
210	14.0
50	13.5
20	12.7
10	12.3
1.8	11.5
0.7	11.0

we propose (Fig. 6) that the two duplex GAA·TTC tracts in the direct repeat orientation (GAA repeats are 5′ → 3′ in the same DNA strand) enable formation of an R·R·Y triplex. Prior investigations (20) favor the R·R·Y triplex. Second, previous investigations (19, 22, 27, 33) revealed that the R·R strands are antiparallel in a stable triplex in the presence of divalent metal ions. Third, we presume that the DNA topology in solution is plectonemic (48) rather than solenoidal. For the previous model (20) which hypothesized the intermolecular interaction between two triplexes, the orientation between the two triplexes could be in either direction. However, the results presented here clearly demonstrate that the sticky DNA structure is formed intramolecularly and not intermolecularly. Furthermore, the single, long triplex model (Fig. 6) is substantially more thermodynamically plausible than the bitriplex model (20) because it contains no regions of unpaired nucleotides.

Influence of the Length of $(GAA{\cdot}TTC)_n$ Tracts on the Length of the DNA/DNA Interacting Regions—Investigations were conducted by EM to determine the influence of the length of the TRS on the lengths of the DNA/DNA interacting regions to obtain further information about the structural details of sticky DNA. By measuring the lengths of the DNA/DNA associated regions by EM as a function of the known lengths of the GAA·TTC tracts (from 75 to 270 repeats) for four plasmids, we wished to obtain further information on whether sticky DNA is a single long triplex (Fig. 6) or a strand-exchanged bitriplex (20). In the former case, at least some of the DNA/DNA associated regions as seen by EM would be longer than half the known length of the TRSs (from DNA sequence analyses). However, in the latter case, the maximum length that should be observed by EM is half the known length of the TRS because the intermolecular, strand-exchanged bitriplex structure can only be half the length of the TRS tract because of their folded-back intramolecular nature (20).

EM studies were conducted on plasmids containing $(GAA{\cdot}TTC)_n$ tracts where n = 75, 150, 190, 230, and 270 (Fig. 1). Head-to-tail biological dimers of each of these plasmids were isolated as described previously (see "Experimental Procedures"). The plasmids were linearized by restriction enzyme cleavage to evaluate the presence of sticky DNA and were analyzed by EM after cytochrome *c* spreading. The restriction enzymes used were the following: for pRW3822 and pRW3823, *Eco*NI, *Eco*RI, *Pst*I, *Sac*I, *Sap*I, and *Xmn*I; for the other three DNAs, *Eco*RI. Identical results were obtained for all restriction enzymes for a given DNA.

The composite results for the measured lengths of the DNA/DNA interacting regions for the inserts over a 3.6-fold range of lengths are shown in Fig. 7 and Table III. In general, the DNA/DNA interacting regions were 20–50% of the known length of the GAA·TTC tracts. However, especially for the shorter GAA·TTC tracts (75 and 150 in length), a substantial percentage of the molecules had interacting regions that were longer than half the known length of the TRS. For the longer TRS tracts (230 and 270 repeats), a smaller percentage of the DNA/DNA interacting regions had a length greater than half of

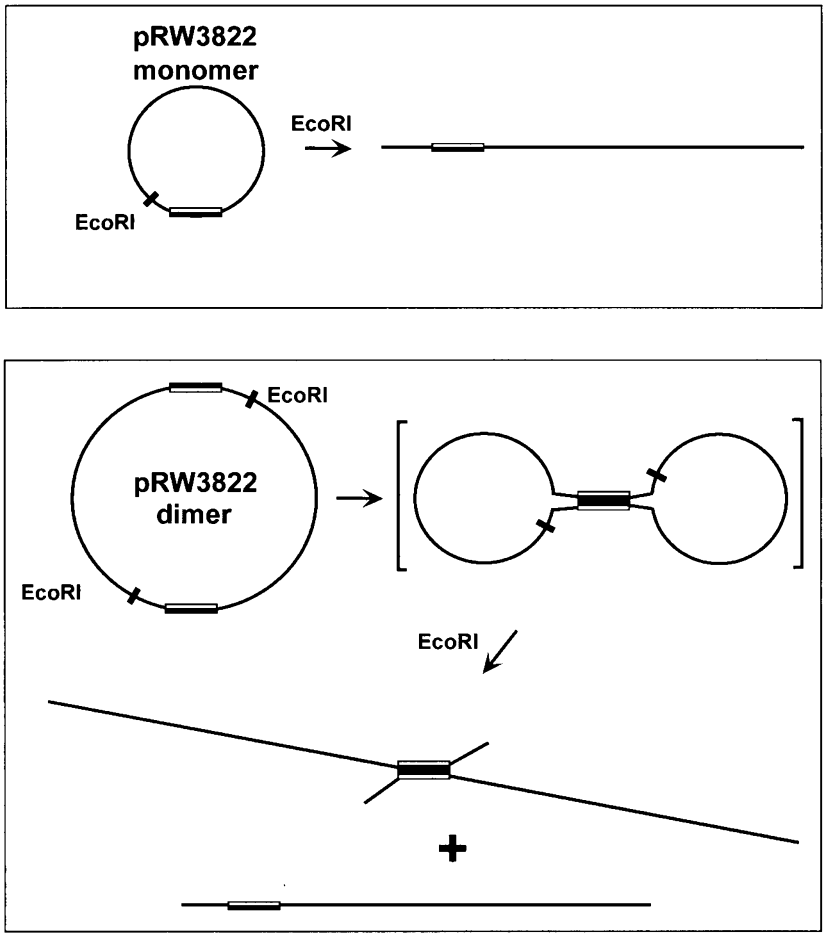


FIG. 3. **Schematic representation of the results from the data shown in Fig. 2.** RB formation was observed only when more than one GAA·TTC tract (dimer) was present in the supercoiled DNA molecule.

TABLE II
Amount of RB formed from plasmid dimers as a function of TRS length, orientation, and sequence

To compare the yield of RB formed from plasmid dimers containing different lengths and orientations of $(GAA{\cdot}TTC)_n$ and harboring different extents of (GGA·TCC) substitution, we studied purified dimers of pSPL3 derivatives, except pRW4204, where the vector was pGEM-3Zf(−). The definition of the orientation of the inserts has been published previously (18). Cleavage by *Eco*RI (see "Experimental Procedures") was conducted for the set of plasmid dimers under the same conditions. Yields of RB (in percent) were compared with the amount observed from purified dimers of pRW3822 under the same conditions. All plasmids had the superhelical density as isolated from *E. coli* (−0.05) (39–41). In parallel studies, the isolated monomeric forms of all eight plasmids were studied; no RB was found in any case.

Plasmid	Length of $(GAA{\cdot}TTC)_n$	Orientation of insert	Percent of (GGA·TCC) substitution	RB formation
	n		%	%
pMP142	33	I	0	0
pRW3821	59	I	0	24
pMP178	75	I	0	67
pRW3828	190	II	0	100
pRW4204	150	II	0	100
pMP193	130	I	50	0
pRW2113	131	I	20	67
pRW4222	136	I	11	74

the known TRS. However, even in these cases, 34 and 10%, respectively, of the total population of molecules had lengths longer than half the known length of the TRS tract (Table III). That a smaller percentage of the molecules with the longer TRS would have long DNA/DNA interacting tracts is as expected because the supercoil density for all plasmids was the same (*e.g.* as isolated from *E. coli*, approximately −0.05) (39–41). Parallel but limited studies with pRW3828 $[(GAA{\cdot}TTC)_{190}]$ gave results consistent with the data in Fig. 7 and Table III (data not shown). In summary, these data provide strong and direct evidence that sticky DNA is one long triplex molecule.

Influence of Negative Supercoil Density on the Length of the DNA/DNA Interacting Regions—Previous studies (20) demonstrated the necessity of a critical level of negative supercoil density for the formation of RB in preparations of pRW3822. We demonstrated previously (20) that addition of $-0.025 \pm 10\%$ supercoil density to the native value increases the yield of RB by ~30%. The molecular basis of this stability was attributed to the stabilization of DNA triplexes within the GAA·TTC tract (19, 26–29) which then participated in the formation of sticky DNA.

Considering the investigations described herein with plasmids containing two GAA·TTC tracts, we reasoned that an evaluation of the influence of negative supercoil density on the length of the sticky region as measured by EM could be critical to discriminate between the single, long triplex model (Fig. 6) *versus* the intermolecular bitriplex model (20). For the single, long triplex model, the addition of negative supercoil turns in pRW3822 might cause an elongation of the DNA/DNA interacting regions, which may be determined by EM. This rationale is outlined schematically in Fig. 8. Alternatively, for the intermolecular bitriplex model (20), the addition of negative supercoil turns in pRW3822 should lead to a more complete formation of the individual folded-back intramolecular triplexes in the GAA·TTC tracts, thus maximizing the EM observed length at half the length of the 450 bp of the TRS tracts.

To evaluate these questions, we prepared samples of pRW3822 (Fig. 1) which contained different levels of negative supercoil density. To prepare relaxed molecules (average su-

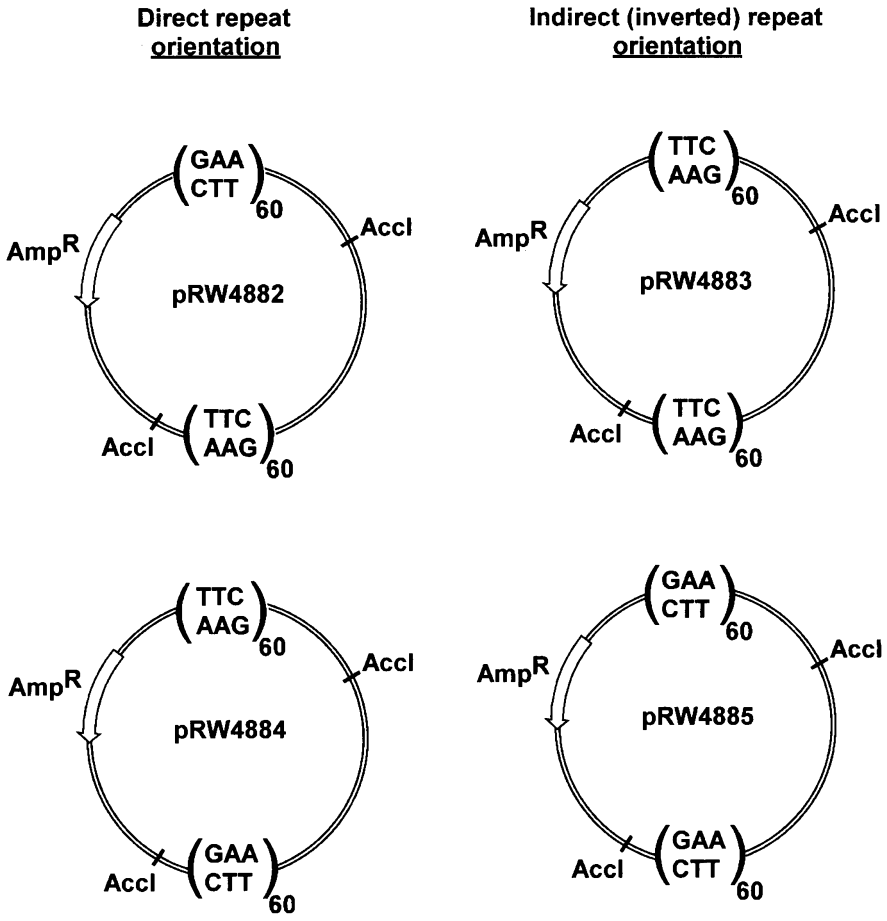


FIG. 4. **Schematic diagram of plasmids containing two GAA·TTC tracts used to determine the influence of the insert orientation on RB formation.** The length of the plasmids is 4,888 bp; the vector is pBR322. The plasmids have two *Acc*I recognition sites separated by the GAA·TTC tracts.

percoil density = 0.0), pRW3822 was treated with topoisomerase I from calf thymus in the presence of Mg^{2+}; also, the DNA was treated with topoisomerase I from chicken erythrocytes in the absence of divalent metal ions. Both preparations were completely relaxed as monitored by agarose gel electrophoresis. EM studies on these relaxed DNA preparations (Table IV) showed a complete disappearance of the X-shaped dimeric molecules that are characteristic of sticky DNA (20).

Alternatively, when the negative supercoil density was increased by −0.025 over the density as isolated from *E. coli*, a substantial increase (1.4-fold) in the average length of the DNA/DNA region was observed for the median values (Table IV). In fact, the average value for the length of the DNA/DNA interacting region is shifted from half the known length of the $(GAA{\cdot}TTC)_{150}$ tract (450 bp) to 62%, *i.e.* a 25% increase. Hence, these results also provide strong EM evidence for the single long triplex model for sticky DNA and are clearly not in agreement with the intermolecular bitriplex model (20) (see "Discussion").

Parenthetically, in these investigations at all negative supercoil densities, no molecules were observed containing bulges, bubbles, or bends in the sticky DNA regions.

Degree of Bending of the DNA/DNA Interacting Region—Measurements were conducted on the degree of bending of sticky DNA to discriminate further between the two models. The single, long triplex model for sticky DNA should be very stiff, in fact, stiffer than duplex B-DNA (49). Alternatively, the intermolecular bitriplex model (20) may be flexible because of some unpairing of bases at the interface between the two triplexes (20). The degree of bending of DNA is an index of DNA stiffness (45). The EM technique used for these investigations (see "Experimental Procedures") was developed for the meas-

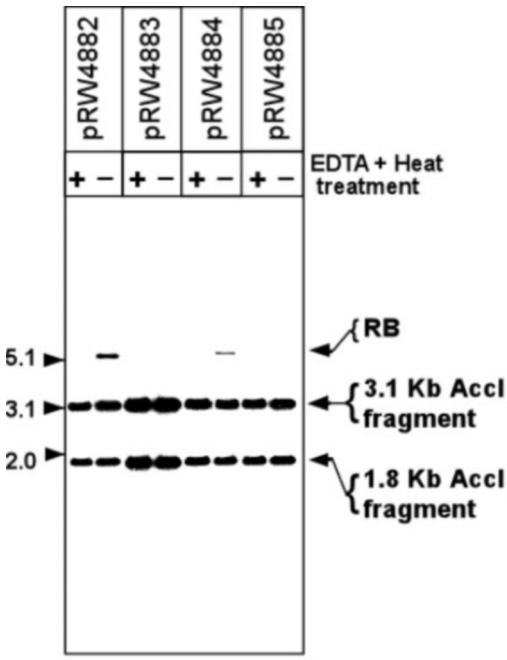


FIG. 5. **Determination of the influence of the insert orientation on RB formation.** Plasmids (Fig. 4) were cleaved with *Acc*I and analyzed on a 1% agarose gel after end labeling (see "Experimental Procedures"). To determine RB formation, after cleavage each sample was divided in two aliquots. One of the aliquots underwent heat treatment (70 °C in the presence of 50 mM EDTA for 10 min) to determine the presence or absence of the dimer-associated DNA molecule (sticky DNA) (see "Experimental Procedures"). The other portion was untreated.

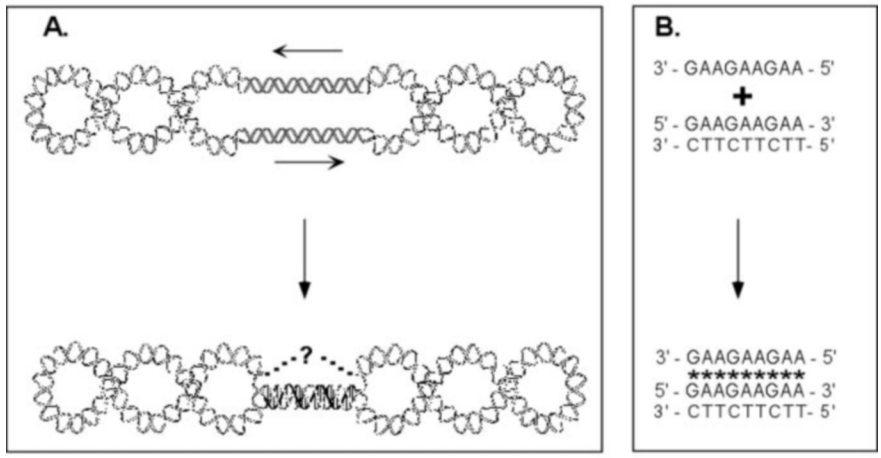


FIG. 6. **Schematic representation of the possible events taking place during the interactions between two antiparallel GAA·TTC regions in one plasmid.** *A*, emphasis is focused on the behavior of the two GAA·TTC tracts in the direct orientation in one plasmid. The *question mark* indicates that the structure of the unpaired polypyrimidine strand is uncertain. The GAA·TTC duplex regions are shown as *straight*, not interwound, helices for the sake of clarity. The model is based on the knowledge that the two purine strands in an R·R·Y triplex are antiparallel (Refs. 50 and 51; for review, see Ref. 33). *B*, proposed base pairing in GAA·GAA·TTC triplex. *Stars* designate Hoogsteen pairs. A detailed description of the triple base interactions in all three reading frames has been published previously (22).

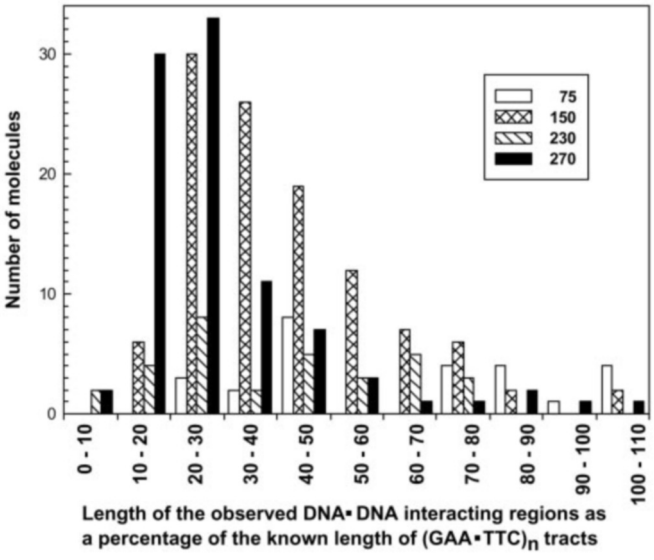


FIG. 7. **Determination by EM of the influence of the length of GAA·TTC repeats on the length of the DNA/DNA interacting regions.** To compare the lengths of the DNA/DNA interacting regions, plasmids pMP178, pRW3822, pRW3827, and pRW3823 which contained $(GAA{\cdot}TTC)_n$ where n = 75, 150, 230, and 270, respectively, were investigated. The plasmids were linearized by restriction enzyme cleavage and analyzed by EM after cytochrome *c* spreading. The restriction enzymes used were the following: for pRW3822 and pRW3823, *Eco*NI, *Eco*RI, *Pst*I, *Sac*I, *Sap*I, and *Xmn*I; for the other two DNAs, *Eco*RI. No statistical differences in the length distributions were found if the composite data for all enzymes were plotted *versus* the data for each of the separate enzymes.

urement of the degree of bending (45).

A simple measure of stiffness applied in the past involves measuring the straight line distance between the ends of a DNA or RNA segment and dividing this by the curvilinear length of the segment ($E - E/L$) (45). A very stiff DNA will exhibit mean values near 1.0 and highly bent DNAs, such as those bent by phased tracts of adenines, values closer to 0. Here, the plasmid pRW3822 was cleaved with *Eco*RI placing the region of sticky DNA 5.6–9.9% from the nearest ends of the two short DNA arms of the X-shaped molecule. For 24 examples, the $E - E/L$ value for the region of sticky DNA was 0.934 ± 0.078 (Table V). As a direct comparison, for each molecule, a DNA segment of the same length as a sticky segment was selected 5.6–9.9% in from one end of a long arm and the $E - E/L$ value determined. Here, the value was 0.885 ± 0.142 (Table V), showing that indeed the sticky DNA is significantly stiffer than an equivalent length of duplex DNA in the same environment. For further comparison, previously determined values for similar lengths of pBR322 and highly bent kinetoplast DNAs are shown. These comparisons show that sticky DNA is significantly stiffer than normal B form DNA and much less bent than highly curved DNA of the same approximate length. Thus, these DNA stiffness measurements provide a third direct determination by EM that sticky DNA is a single, long triplex. Fig. 9 shows a typical X-shaped molecule that is characteristic of sticky DNA. Also, no molecules were observed which contained bulges or bubbles in the sticky DNA region.

TABLE III
Dependence of the length of the DNA/DNA interacting region on the length of the $(GAA{\cdot}TTC)_n$ repeat tracts

To compare the lengths of the DNA/DNA interacting regions, plasmids pMP178, pRW3822, pRW3827, and pRW3823, which contained $(GAA{\cdot}TTC)_n$ where n = 75, 150, 230, and 270, respectively, were investigated. The plasmids were linearized by restriction enzyme cleavage and analyzed by EM after cytochrome *c* spreading. The restriction enzymes used were the following: for pRW3822 and pRW3823, *Eco*NI, *Eco*RI, *Pst*I, *Sac*I, *Sap*I, and *Xmn*I; for the other two DNAs, *Eco*RI.

$(GAA{\cdot}TTC)_n$	No. of molecules scored	Average length of the DNA/DNA interacting region as a percentage of the length of $(GAA{\cdot}TTC)_n$ tract	Percent of molecules with DNA/DNA interacting regions with length greater than $n/2$
n		%	%
75	26	63	50
150	112	41	26
230	32	40	34
270	92	29	10

DISCUSSION

Sticky DNA is an X-shaped molecule found by EM in plasmid preparations containing long GAA·TTC tracts (greater than or equal to 59 repeats in length) after linearization of the plasmid by cleavage at a unique restriction site (20). Sticky DNA was also detected by agarose gel electrophoresis after linearization of the plasmid; gel retardations as large as 6-fold (to 42 kbp from 7.1 kpb) were observed when as little as 6% (450/7,100 bp) of the linearized plasmids were associated at the long GAA·TTC tracts. The extent of the retardations was related to the distance between the linearization sites and the GAA·TTC tract. Negative supercoil density stabilized the R·R·Y triplexes at neutral pH. In fact, low pH destabilized the sticky DNA conformation. Divalent metal ion is required for stabilization of sticky DNA, which can be dissociated by heat treatment in the presence of EDTA (70 °C for 10 min in 1 mM EDTA). EM studies (20) rigorously demonstrated that the crossover point for the sticky DNA structure was at the GAA·TTC repeat tracts. Hence, we postulated (20) a model for the association of two triplexes formed in independent plasmids at the

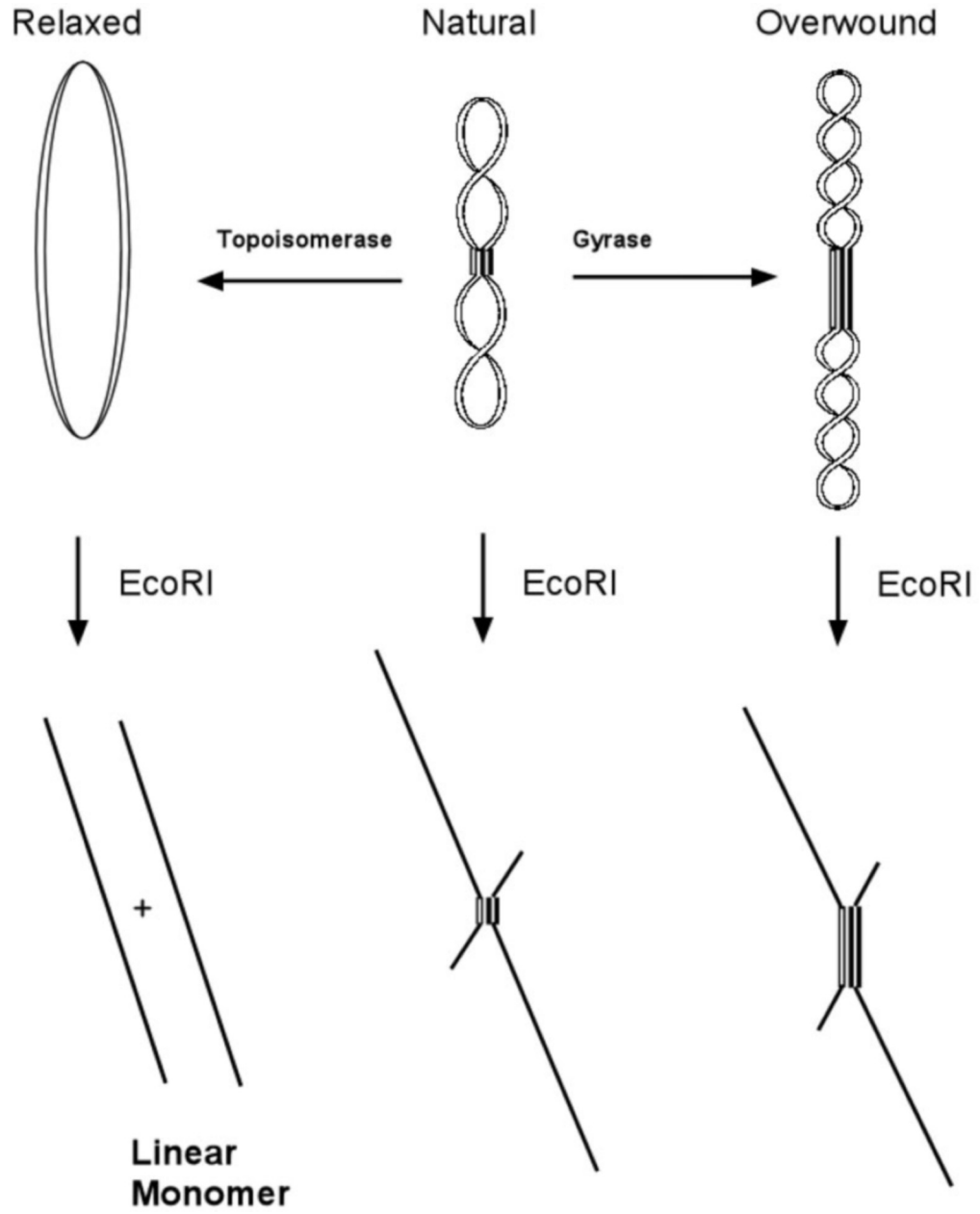


FIG. 8. **Schematic representation of the effect of negative supercoiling on the length of the DNA/DNA interacting regions.** The model shows the influence of the changes in the negative superhelical density. Average negative densities were 0.0 for relaxed, −0.05 for natural (39–41), and −0.075 for negatively overwound DNA (see "Experimental Procedures").

TABLE IV
Quantitation of the length of the DNA/DNA interacting regions observed by EM for dimeric associated DNAs

To compare the length of the DNA/DNA interacting regions for pRW3822 relaxed by topoisomerase I, at the native superhelical density and with an additional $-\sigma = 0.025$ added by DNA gyrase (see "Experimental Procedure"), samples of DNA were linearized by *Eco*RI and analyzed by EM after cytochrome *c* spreading. The statistical significance of the difference in the values for the average length pass a *t* test at $p < 0.05$. For pRW3822, which was completely relaxed by topoisomerase I in either the presence or absence of Mg^{2+}, RB was not detected either by EM or gel electrophoresis.

Amount of supercoiling	Length of the DNA/DNA interacting region (bp)		No. of molecules scored
	Average (± S.D.)	Median	
0.0	NA[a]	NA	Not observed
−0.05	183 ± 80	161	80
−0.075	280 ± 97	292	28

[a] NA, not applicable.

long GAA·TTC tracts to explain the extreme stabilization of the sticky DNA structure.

Herein, we have extended our structural studies on sticky DNA. These new investigations demonstrate that this novel DNA structure is formed only when two tracts of long GAA·TTC repeats are present within one plasmid molecule; no sticky DNA is formed when a single GAA·TTC tract is present. Thus, the formation of sticky DNA must be derived from an intramolecular association of the two TRS tracts effected by slithering of the plectonemic supercoiled plasmid. Hence, the two tracts of GAA·TTC align with each other and form a single long triplex, probably within living *E. coli* cells. Because we have no evidence for the formation of sticky DNA from plasmids containing a single tract of GAA·TTC, we believe that our prior hypothesis of the strand exchange structure between two intramolecular triplexes (20) should be modified. It should be noted that sticky DNA is the first non-B form DNA conformation that requires the presence of two parental sequences (*i.e.*

GAA·TTC) in one plasmid DNA.

Our current model for sticky DNA as a single long triplex formed intramolecularly is based on the following considerations. First, sticky DNA is formed only from biological dimers of plasmids which contain two tracts of GAA·TTC or from recombinant plasmids that were constructed to contain two tracts of GAA·TTC. Sticky DNA has never been observed from plasmids rigorously purified to contain only a single GAA·TTC tract. Furthermore, the retarded band (sticky DNA) is only found when the two GAA·TTC are in the direct repeat orientation. If the tracts are in the indirect orientation, no sticky DNA has ever been observed. This is a critical result because the two purine strands must be in the antiparallel arrangement (20, 50, 51) for formation of a stable R·R·Y triplex. For all of these investigations, we presume that the topology of the plasmid is plectonemic in solution (39).

Second, the yield of RB was essentially independent of the concentration of the DNA, supporting the notion that a unimolecular reaction takes place intramolecularly within a single DNA circular molecule. Third, EM length determinations of the DNA/DNA interacting region in sticky DNA revealed a statistically significant number of hybrid molecules that were longer than half the known length of the GAA·TTC tract. Because the strand-exchanged bitriplex model (20) consists of two folded-back intramolecular triplexes, the maximum length observable by EM would be half the length known from DNA sequence studies. Thus, this result strongly supports the notion of a single long triplex.

Fourth, EM studies were conducted on plasmids containing the GAA·TTC tracts prepared at different supercoil densities. At higher negative supercoil densities, the average length of the DNA/DNA interacting region was extended, as expected, approaching the known length of the triplet repeat tract. This result is expected because pRW3822 contains 450 bp (*i.e.* ~42 helical turns) of GAA·TTC and thus would require the relaxation of ~40 supercoil turns to convert the duplex repeat tract fully into the extruded triplex. In the case of the intermolecular strand exchange model (20), the opposite behavior should be observed because at higher negative supercoil densities, further triplex extrusion would be expected, and the maximum length observed for the DNA/DNA interacting region should approach half of the known length of the TRS tract because of their folded-back structures. Also, EM determinations revealed that the region of association (the triplex segment) was substantially stiffer than other duplex segments of the same length within the same molecule. We would expect that sticky DNA should be significantly stiffer than B-DNA because of the presence of a triplex and the absence of flexible junctions within the DNA/DNA associated region. Alternatively, the strand-exchanged bitriplex model (20) could be flexible. Furthermore, no bulges, bubbles, or flexible regions were observed within the sticky DNA tract as revealed by EM, as expected for a single long triplex model, but they could, in fact, be present for the less thermodynamically plausible strand-exchanged intermolecular model (20).

An uncertainty concerning the single long triplex model that is formed intramolecularly is the fate of the "unpaired" poly(Y) strand, which is not engaged in Watson-Crick base pairing. At present, we have no additional experimental results beyond our P1 nuclease mapping studies reported previously (20–22). We presume that this poly(Y) sequence is wrapped around the R·R·Y triplex in a nonorganized way but trails the orientation of the poly(R) strand with which it had been paired previously.

Sticky DNA is formed under physiological conditions, and the potential role of this DNA structure in the etiology of FRDA has been reviewed (20–22). Because we have demonstrated herein that two long GAA·TTC tracts react intramolecularly to form the sticky conformation, it is possible that these TRS regions in the daughter strands behind the replication fork could associate to form the single long triplex, which would

TABLE V

Degrees of bending for pBR322, kinetoplast DNA, and sticky DNA

To compare the degrees of bending in the DNA/DNA interacting region (sticky DNA) with B-DNA from pBR322 and highly bent kinetoplast DNA (45), pRW3822 ($-\sigma = 0.075$) was linearized by *Eco*RI, analyzed by EM after tungsten mounting, and compared with the published data (45) for pBR322 and kinetoplast DNA. In theory, the degree of bending is represented by dividing the straight line distance between two ends of each molecule by its curvilinear length; the value 1.0 represents a straight line, and 0.0 represents a full circle (45). In practice, in our case, we measured this value for the pieces of DNA with the similar curvilinear length no longer than 0.5 kb.

Type of DNA	No. of molecules counted	Degree of bending		
		Average	Standard deviation	Median
Kinetoplast DNA	56	0.563	0.246	0.570
pBR322	70	0.855	0.142	0.900
Sticky DNA	29	0.934	0.078	0.958

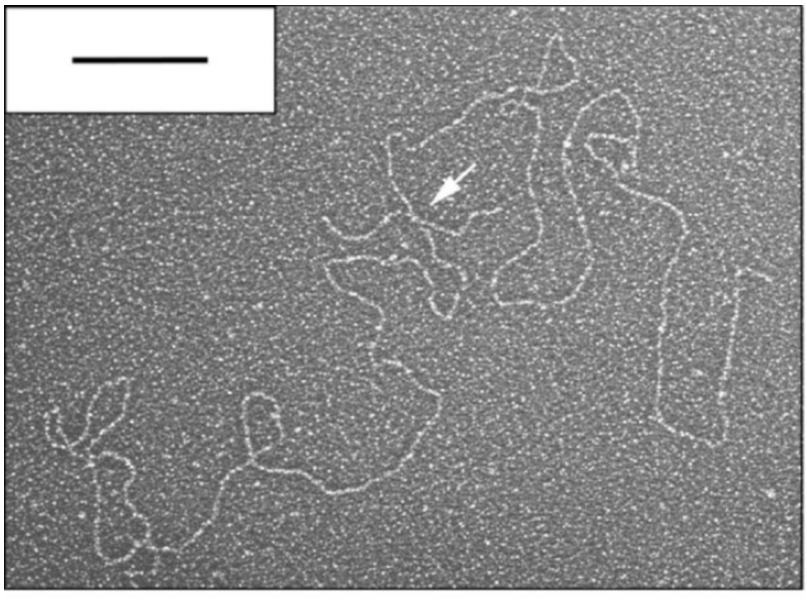


FIG. 9. **Example of a plasmid dimer cleaved to place the region of sticky DNA near one end.** The DNA was mounted directly onto a thin carbon support and shadowcast with tungsten. The *arrow* reveals the region of sticky DNA. To examine the degree of curvature of this region, the straight line end-to-end distance of the sticky segment was divided by its curvilinear length, and this ($E - E/L$) value contrasted to that for a similar duplex segment the same distance in from one long arm of the X-shaped DNA. In addition, these values were compared with previously determined values for other DNAs prepared by the same EM method (Table V). The *bar* is equivalent to 0.5 kbp.

inhibit transcription (18, 22). The relatively high level of negative supercoil density (52) behind the replication fork would enhance the triplex sticky DNA formation. Thus, the presence of sticky DNA in an FRDA patient would inhibit transcription and result in reduced levels of frataxin. Furthermore, it is possible that other intramolecular interactions take place within the FRDA genetic locus between one long tract of GAA·TTC and another, shorter, GAA·TTC tract and/or the $(GAAGGA{\cdot}TCCTTC)_{65}$ region, which is also in intron 1 of the frataxin gene (18, 23). The companion paper (35) reveals the plausibility of these interactions.

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