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Slow cruciform transitions in palindromic DNA

(DNA supercoiling/self-complementary DNA/hairpins)

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ABSTRACT **Extrusion of cruciform structures in self-complementary regions of DNA is known to be favored by negative supercoiling of DNA. We show here that, in moderately supercoiled DNA, cruciform extrusion is a very slow process. In plasmid pUC7 DNA, with a 48-base-pair palindrome, the half-time of extrusion at 50°C is typically several hours; rates are even slower at lower temperature. The rates increase significantly with increasing DNA supercoiling but are only slightly faster in DNA species with much longer palindromes. The reabsorption of cruciform arms is also very slow. The equilibrium between cruciform and regular DNA structures is sensitive to changes in the linking number. Measurement of this equilibrium leads to an estimate of 18 kcal/mol (75.3 kJ/mol) for the free energy required to generate a cruciform structure. In bacterial cells, cruciform DNA may be rare, even when it is thermodynamically favored, because of its slow formation.**

Palindromic sequences occur frequently in DNA and are often found at functionally interesting locations such as replication origins or operator sites. It was recognized early that palindromic (self-complementary) regions of DNA can exist in two alternative structures—the regular DNA duplex with base pairing between strands, and a cruciform structure with intrastrand base pairing of the self-complementary sequence (1, 2). Particularly since the suggestion that cruciform structures would be favored by negative supercoiling of DNA (3), there has been interest in the stability of cruciform DNA, with a view to assessing its possible intracellular role. An investigation of circular DNA with a very long perfect palindromic sequence constructed from plasmid pBR322 showed that cruciform arms were generated when the DNA was supercoiled, as seen either by electron microscopy or by altered electrophoretic mobility (4) or by the DNA's insensitivity to a restriction endonuclease whose site is rendered single-stranded at the tip of a hairpin (5). This DNA was also extremely inefficient in transforming *Escherichia coli*, even when introduced in a relaxed and therefore non-cruciform structure, implying that long palindromes may generate cruciform structures intracellularly that are incompatible with survival of the DNA. Shorter palindromes are found in naturally occurring plasmid DNA species and evidently do not inhibit maintenance of the plasmid. It has been shown that the centers of several short palindromic sequences (24 to 30 base pairs) found in the plasmids pBR322 and ColE1 are a preferred target for single-strand-specific nucleases, and it was suggested that cruciform structures present before the nuclease challenge were being detected (6, 7).

In this work, we studied in more detail the extrusion of cruciform arms in palindromes of various lengths. The surprising result is that, in moderately supercoiled DNA, the transitions are exceedingly slow. The half-time of extrusion can be many hours at physiological temperature; once generated, cruciforms are also very slow to be reabsorbed. These kinetic factors may be important in determining the intracellular state and function of palindromic DNA.

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MATERIALS AND METHODS

DNA Samples. Plasmid pUC7 DNA (8) contains a 48-base-pair palindromic insert and is 2.7 kilobase pairs (kb) long. Plasmid pUC8 DNA, which has a nonpalindromic insert in the same vector (8), was used as a control. Plasmid pMK204 was constructed by inserting into the *Hin*dIII site of the related plasmid pUC9 (8) a 114-base-pair palindromic sequence derived from simian virus 40 DNA: nucleotides 5,170 to 5,226 to 5,170 (9) in head-to-head orientation. Plasmid pMM110 DNA (10) was cut with either *Eco*RI or *Bam*HI, and the larger fragment was religated. The resulting circular DNA species, which we call pMM110R or pMM110B, respectively, contained palindromes of 7.4 or 6.7 kb. These two DNA species could not be propagated intracellularly because of the long palindromic regions derived from pBR322 (4).

Plasmid DNA was prepared by a standard method (11). DNA samples with various degrees of supercoiling were prepared by relaxing the DNA with a chicken reticulocyte nuclear extract (12), with suitable concentrations of ethidium bromide added. Single topoisomers of pUC7 and pMK204 were purified as described (13). Their linking number, relative to the average linking number of a DNA sample relaxed at 37°C, was measured by the method of Keller (14). Topoisomers are indexed by the negative of their linking difference from relaxed DNA; for example, T_{12} has a linking difference of -12 (± 0.3). The values of specific linking difference also refer to 37°C. For measurements of *in vivo* specific linking difference, *E. coli* strain N100 (*recA*) carrying, respectively, pUC7, pUC9, or pMK204 was grown at 37°C in LB medium (per liter: 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl) to a cell density of 5×10^8/ml. Plasmid DNA was prepared as above.

Enzymes. *Eco*RI, *Bam*HI, *Pst* I, and *Pvu* II (New England BioLabs) were used according to the manufacturer's instructions, except when the enzyme was used to test for the presence of cruciforms, in which case the reaction temperature was lowered to 20°C to avoid further rearrangement of DNA during the assay. T4 endonuclease VII (15) was the gift of B. Kemper.

Other Methods. Gel electrophoresis was carried out in 0.8% or 1.2% agarose gels, in 50 mM Tris/33 mM H_3PO_4/1 mM EDTA, pH 7.5 (TPE buffer), with varying concentrations of chloroquine added (16). The temperature was 5–10°C. After soaking in buffer to remove chloroquine, gels were stained with ethidium (1 μg/ml) and photographed. Bands were quantitated with a Joyce–Loebl densitometer. Incubations for cruciform extrusion were carried out in 10 mM Tris·HCl, pH 8.0/1 mM

Abbreviation: kb, kilobase pair(s).

EDTA (TE buffer) with varying concentrations of NaCl added or in TPE buffer. Temperature-dependence experiments were done in TE containing 0.03 M NaCl or in the same medium with Tris replaced by 10 mM 4-morpholinepropanesulfonic acid (Mops) buffer at pH 7.9 (the pH of Tris buffer varies rather steeply with temperature). Identical rates were obtained in both buffers between 24°C and 50°C.

RESULTS

When a cruciform structure is extruded from negatively supercoiled DNA (Fig. 1), the DNA is partly or entirely relaxed, depending on the length of the palindromic region, and therefore migrates more slowly in gel electrophoresis. To avoid confounding the cruciform species with the normal topoisomer series, it is convenient to display all species in two-dimensional gel electrophoresis (17). Fig. 2 presents such an experiment with pUC7 DNA. If conditions are adjusted so that some cruciforms are present in the first (vertical) dimension but are largely annealed out by partial relaxation of the DNA with chloroquine before electrophoresis in the second (horizontal) dimension, the electrophoretic pattern of pUC7 DNA shows a spur, due to molecules with cruciforms, in addition to the arc of normal topoisomers. In comparison, pUC8 DNA, which has a nonpalindromic insert in the same vector, shows no spur.

Preliminary experiments with the two-dimensional gel method showed that cruciform structures were generated extremely slowly at low temperature (little or no conversion in several weeks at 4°C) and even remarkably slowly at 50°C, with half-times of several hours. In order to study these rates in more detail, it was necessary first to find out how they varied with ionic conditions. Two DNA samples were examined: a sample of pMM110R with an average specific linking difference of −0.048, and the single topoisomer T_{13} of pUC7 DNA (specific linking difference, −0.050). The rate of cruciform extrusion at 50°C passed through a sharp maximum when the NaCl concentration was varied, the optimum being between 0.03 and 0.04 M for both DNAs (Fig. 3). Even in the absence of NaCl, the ionic strength of the buffer components (about 10 mM) is sufficient to ensure that at 50°C the DNA is well below its denaturation temperature. At still lower salt concentrations, anomalous behavior could be expected.

Approximately the same maximal rate was obtained in the TPE buffer (without added salt) used for gel electrophoresis, a point of convenience for later experiments. Among several multivalent ions that were tested as accelerants of extrusion, the most effective was spermidine; when added to TE buffer/0.01 M NaCl at a concentration roughly equivalent to the DNA charge (e.g., 10 μM spermidine with a DNA solution 25 μM in nucleotides), it stimulated the extrusion rate about 3-fold above that at the optimal NaCl concentration. Other multivalent cations (e.g., Mg^{2+}, $Co(NH_3)_6^{3+}$) were relatively ineffective.

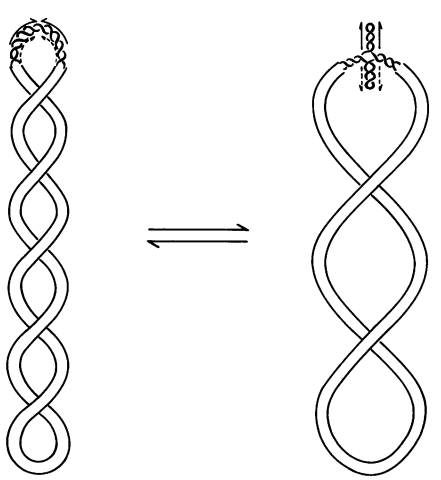

FIG. 1. Extrusion of a cruciform structure in negatively supercoiled DNA leads to decreased supercoiling.

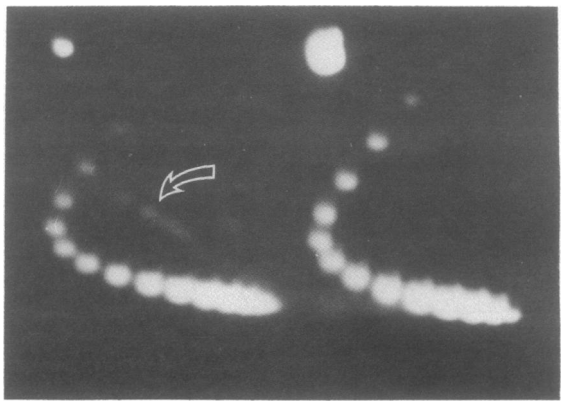

FIG. 2. Demonstration of a cruciform structure in pUC7 DNA (*Left*) by two-dimensional gel electrophoresis. The cruciform species is seen as a spur (arrow) superimposed on the topoisomer pattern. The control plasmid pUC8 (*Right*), lacking the palindrome, does not show a spur. Both DNA samples had an average relative linking difference of −0.06. They were incubated at 50°C for 30 min in TE buffer/20 μM spermidine before electrophoresis, to allow some cruciform extrusion. The gel contained 1.5 μg of chloroquine per ml during electrophoresis in the first (vertical) dimension. It was then soaked at 20°C for several hours in TPE buffer containing 6 μg of chloroquine per ml and at 50°C for 30 min before electrophoresis in the second (horizontal) dimension in the same buffer. The intense spot at the upper left of each pattern is nicked circular DNA.

The extrusion rate increases significantly with increasing negative supercoiling. The experiment in Fig. 4 examines the rate of extrusion of pUC7 topoisomers T_{12}, T_{13}, and T_{14} at 50°C. The DNA in the fastest (supercoil) band is slowly converted to a slower band of cruciform DNA. When measured against a ladder of pUC7 topoisomers (not shown), the difference in mobility corresponds to a linking number change of +4.5 (±0.3) which is close to the expected relaxation caused by extruding the 48-base-pair palindromic region. [We have previously shown

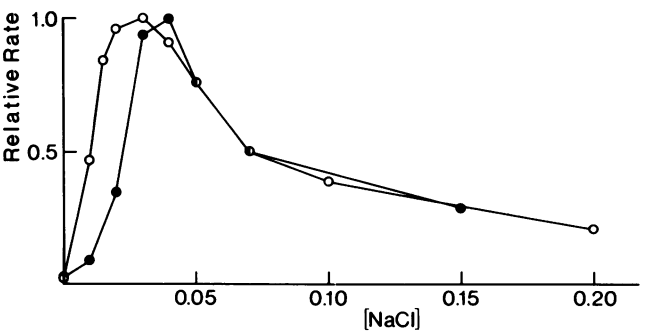

FIG. 3. Variation of cruciform extrusion rate with NaCl concentration. pUC7 DNA topoisomer T_{13} (○) and pMM110R DNA (●) with an average specific linking difference of −0.048 were incubated at 50°C in TE buffer containing the indicated NaCl concentrations for 3 hours (pUC7) or 1 hour (pMM110R). The pUC7 DNA was then electrophoresed on a gel containing 1.5 μg of chloroquine per ml, and the fraction of cruciform DNA was measured. The pMM110R DNA samples were digested (all at the same salt concentration) with an excess of *Eco*RI and the fraction of resistant material (cruciform DNA) was quantitated by gel electrophoresis. The rates for each DNA have been separately normalized to 1 at the maximum.

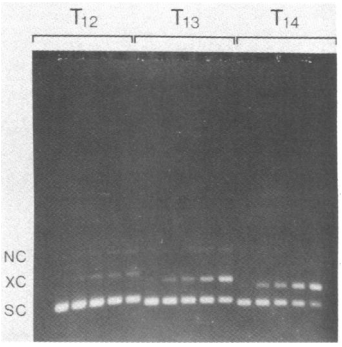

FIG. 4. Cruciform extrusion rates of different pUC7 topoisomers. Topoisomers T_{12}, T_{13}, and T_{14} were incubated at 50°C in TPE buffer for 0, 30, 60, 120, or 240 min. Electrophoresis was in 1.2% agarose gel containing chloroquine at 1.5 μg/ml. The position of cruciform (XC), regular supercoiled DNA (SC), and nicked circular DNA (NC) are indicated.

that the existence of a small cruciform itself does not significantly alter the gel mobility of a circular DNA (4, 5).] Over the range of topoisomers examined here, the rate increased about 3-fold when the linking number decreased by 1 (initial rate was approximately 0.06, 0.18, and 0.43 hr^{-1} for the three samples). Because the pUC7 palindrome is centered on a *Pst* I site, cruciforms could also be quantitated as the fraction of DNA resistant to *Pst* I digestion. Essentially identical rates were obtained by this method (not shown). After 4 hours, none of the extrusion reactions was complete; additional data collected after 24 hours show that the relative amounts of cruciform DNA were: T_{14}, >98%, T_{13}, 95%; T_{12}, 50%; T_{11}, ≈ 2%. To interpret these results, it was necessary to learn whether they represented approximate equilibrium values or continuing slow reactions. Therefore, we devised an experiment in which the final equilibrium state could be approached from samples entirely in the regular or in the cruciform state. Of the four topoisomers used in this series of experiments, only T_{12} yielded adequate proportions of both products.

The equilibration experiment (Fig. 5) consisted of preincubating T_{12} to generate roughly equal amounts of cruciform and regular DNA and then carrying out electrophoresis in one dimension to separate the two forms. Before electrophoresis in the second dimension, the gel was incubated for 36 hr at 50°C to reequilibrate both species. (In control experiments, we have found that the extrusion rate of DNA in agarose gels is similar to that in free solution.) The second electrophoresis revealed that, in addition to some nicking of DNA during the long incubation, a roughly equimolar mixture of cruciform and regular

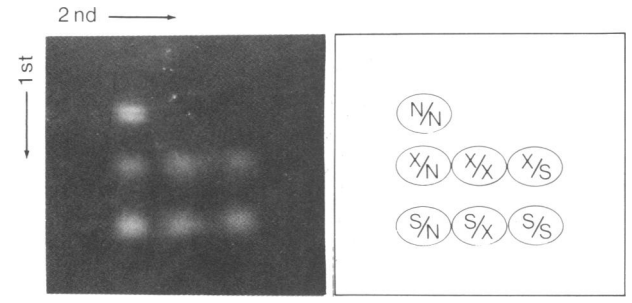

FIG. 5. Equilibrium between cruciform and regular DNA, visualized by two-dimensional gel electrophoresis. A sample of pUC7 T_{12} DNA containing roughly equal amounts of cruciform and regular DNA was subjected to electrophoresis on a gel containing chloroquine at 1.5 μg/ml in the first (vertical) dimension. The gel was soaked in TPE buffer to remove chloroquine and then incubated for 36 hr at 50°C in TPE buffer to reequilibrate the DNA species. Electrophoresis in the second (horizontal) dimension was again in buffer containing chloroquine at 1.5 μg/ml. The molecular species are identified in the accompanying drawing; the species in the first dimension are above the diagonal and those in the second dimension, produced by the intermediate incubation, are below the diagonal. S, regular supercoiled DNA; X, cruciform; and N, nicked circular DNA.

DNA (50–60% cruciform) was produced, starting from either species. Because a 24-hour incubation closely approached the same equilibrium (data not shown), the final values attained in the previous kinetic experiment can be interpreted as showing that the equilibrium fraction of cruciforms depends sharply on supercoiling: for T_{11}, <5%; T_{12}, ≈ 50%; T_{13} and more supercoiled species, >95%. For T_{13} and more highly supercoiled species the equilibrium thus strongly favors the cruciform structure, and the incomplete reactions at early times reflect only the slow reaction rate. The rates measured in these experiments are properly interpreted as relaxation rates, in the kinetic usage of the term.

We also studied the rate of extrusion in topoisomer T_{13} of pMK204 DNA (114-base-pair palindrome) and in a mixed topoisomer sample of pMM110R with an average specific linking difference of −0.048, close to that of the T_{13} topoisomer of pUC7 and pMK204. The rates for both DNAs at 50°C were 4-fold faster than for pUC7 T_{13}, with half-times of 1 hour. These rates are still slow despite the great variations in palindrome length, implying that the length of the self-complementary region is not a primary determinant of the extrusion rate. We do not know if this slight change of rate among the different palindrome species is due to the length of the palindrome or to the difference in the sequence at the middle of the palindrome. [In previous studies with the same palindromic region as in pMM110R (5), faster rates of extrusion were found. However, the DNA in those experiments was supercoiled by DNA gyrase to an extent far beyond the supercoiling studied in the present work, and the results are therefore not strictly comparable.]

The temperature dependence of the rate of extrusion of pUC7 topoisomers was also investigated. In doing so, it was necessary to take into account the increase in DNA superhelicity with decreasing temperature (reviewed in ref. 18). This variation has the effect that pUC7 T_{13} at 50°C has the same relative linking difference as T_{12} at 37°C and T_{11} at 24°C. When initial rates of extrusion were compared in this way, the rate was found to decrease by a factor of approximately 10 for each 10°C decrease in temperature. The heat of activation for the extrusion reaction can thus be estimated to be about 50 kcal/mol.

The equilibration achieved in the experiment of Fig. 5 implies that the reverse reaction, from cruciform to regular structure, must also be very slow. For a negatively supercoiled cruciform to revert to a regular supercoiled form, there obviously is a supercoiling energy barrier against which the branch migration has to take place. This in itself will make the reverse reaction slow. However, we have found that even when a DNA segment containing a cruciform structure is cut out of supercoiled DNA, as can be done by restriction enzyme cleavage, the reabsorption of cruciform arms is still very slow. The DNA remains sensitive for some time to digestion by T4 endonuclease VII under conditions in which this enzyme cuts specifically at the base of cruciforms (10). With a 700-base pair cruciform derived from pMM110B DNA, the half-time of reabsorption was several hours at 50°C (data not shown). One possible explanation for the slow rate is that the complementary single-stranded loops at the tips of the two cruciform arms may be involved in base-pairing with each other, thus retarding branch migration.

We have also tested for the presence of cruciform structures in intracellular DNA by measuring the linking number of extracted pUC7 and pMK204 DNA, in comparison with that of pUC9 DNA which lacks an extensive palindrome. Our expectation was that the relaxation caused by cruciform extrusion would be compensated for by renewed supercoiling to restore the DNA to the same level of torsional stress, leading to a specific linking difference decreased for pUC7 DNA (48-base-pair palindrome) by 0.018 and for pMK204 DNA (114-base-pair palindrome) by

0.041. However, the specific linking difference for all three DNA species in their regular structure, measured by band counting in gel electrophoresis (14), was -0.070 ± 0.003, implying that there was no large proportion of cruciform DNA in the cells.

DISCUSSION

We discuss first the equilibrium between cruciform and regular DNA structures. The equilibrium experiment of Fig. 5 allows estimation of the excess free energy, DG_{xc}, intrinsic to the extrusion of a cruciform structure. At equilibrium, ΔG_{xc} is balanced by the decreased free energy of supercoiling of the cruciform molecule (3). If one assumes that the length of the extruded region is exactly that of the self-complementary sequence, as indicated by the shift in mobility of the cruciform species of pUC7, the known energetics of DNA supercoiling (19, 20) lead to an estimate of $\Delta G_{xc} = 18$ kcal/mol.† [More detailed theoretical treatments of cruciform extrusion (21, 22), allowing for the existence of cruciform structures that are shorter than the maximum permitted by the palindromic sequence, do not seem to be necessary in this case. Fig. 4 shows that the cruciform DNA has an apparent linking number as well defined as a topoisomer of regular DNA.]

The same treatment of supercoiling energetics also predicts a steep dependence of the equilibrium fraction of cruciform on linking number. For pUC7 with its 48-base-pair palindrome, if this fraction is 50% for T_{12} (see Fig. 5), it is predicted to be 4% for T_{11} and 96% for T_{13}. These values are in reasonable agreement with the results of long incubations described above. In addition, if ΔG_{xc} is assumed to be independent of the length of the cruciform and of its detailed sequence, one can apply the same considerations to show that longer self-complementary regions, such as the one in pMK204, should extrude cruciforms more readily (in less supercoiled DNA), and we have found that cruciforms can indeed be extruded in pMK204 DNA at lower degrees of supercoiling than in pUC7 (data not shown). However, shorter palindromes would require more supercoiling for extrusion. For example, the equilibrium fraction of the pBR322 "major" cruciform studied by Lilley (6) is estimated to be 50% at a specific linking difference of -0.070, at the high end of the range of supercoiling normally found for plasmid DNA in *E. coli* (relative linking difference, -0.060 to -0.070).

As to the rates of extrusion, the chief point of interest is their extreme slowness at moderate degrees of supercoiling. We found the reaction to be slow in three DNA species with different palindrome lengths and different central sequences, implying that this may be a general situation. If the rate-limiting step is the opening of a small loop at the center of the palindromic region, the large enthalpy of activation ($\approx$50 kcal/mol) indicates that the loop size must be considerable. However, it is not clear that the kinetically critical intermediate is such a simple denatured region.

The dependence of the rate on salt concentration is in fact not entirely consistent with a short denatured region being kinetically critical. Lowering the salt concentration should facilitate this denaturation and should thus increase the extrusion rate. However, the sharp maximum in the rate as a function of NaCl concentration argues against this simple picture, as does the stimulation by low concentrations of spermidine. Spermidine increases the denaturation temperature of DNA and thus will decrease the fraction of DNA locally melted.

A possible reason for the complex dependence of the rate on salt concentration might be that extrusion involves both denaturation and renaturation, in this way resembling the annealing of DNA. An alternative explanation is that the close proximity of four branches of DNA in the cruciform structure could make extrusion very sensitive to electrostatic repulsive forces at low salt concentration.

Increased DNA supercoiling not only favors the existence of cruciforms at equilibrium but also enhances the rate of extrusion. It is reasonable to suppose that the torsional stress of supercoiling favors intermediate conformations on the path to cruciform extrusion and thus lowers the activation energy of the reaction.

More rapid cruciform extrusion has been reported by groups that used single-strand-specific nucleases to test single-strandedness at the tips of cruciform arms (23) or used a crosslinking reagent to trap cruciform arms (24). We think that these data are not strictly comparable with ours because the use of an enzyme or reagent that binds to the product of extrusion may itself speed the reaction considerably. In addition, we have found that the buffer conditions (6, 7, 23) used for S1 nuclease challenge (pH 4.6, with $ZnCl_2$ added) lead to much faster extrusion rates even in the absence of S1 nuclease. For pUC7 DNA at 37°C, the acceleration over our standard conditions is 20-fold (data not shown).

Our results imply that there are at least two reasons why cruciform structures may not form easily in bacterial cells. First, for short palindromic sequences such as those found in pBR322 DNA (total length, $\leq$ 26 base pairs), the fraction of cruciform structure at equilibrium is expected to be low unless the DNA is highly supercoiled, perhaps in excess of the normal physiological value. Second, for somewhat longer palindromes (e.g., pUC7, pMK207), the intracellular equilibrium may favor extrusion but the rate could well be very slow. We note that our most supercoiled topoisomer of pUC7 (T_{14}) has a specific linking difference of -0.055, near the physiological range. Extrapolation of our results to physiological values of temperature, salt concentration, and DNA specific linking difference implies that the half-time of cruciform extrusion could be many hours, unless there is catalysis by some component of the cellular milieu.

These considerations may explain some of what is known about the state of palindromic DNA in bacteria. Plasmids with palindromic sequences up to 120 base pairs long can be propagated without difficulty, and in several cases there is evidence that the palindrome is not in a cruciform state in the cell (refs. 24 and 25, and our data described above for pUC7 and pMK204). It has been pointed out that cruciform junctions are locally identical to Holliday recombinational intermediates, and their processing by enzymes specific for that structure (10) could lead to destruction of the plasmid DNA. Thus, a low rate of cruciform extrusion, combined with destructive processing of the small proportion of DNA with cruciforms, would account for the survival of these plasmids and the absence of cruciforms in them. However, it is also known that plasmids containing long palindromic inserts either cannot be maintained in *E. coli* (4, 26–28) or undergo gross rearrangements (29). We have shown above that cruciform extrusion is still slow in such a DNA (pMM110R), but if the slow rate persists *in vivo* it is evidently not sufficient to allow the survival of the DNA. The different fate of short and long palindromes requires further investigation.

On the other hand, if there are cells that do not destroy cruciform structures, the slow interconversion in both directions between regular and cruciform structure opens the possibility of a "two-state" DNA structural transition, with molecules in either the cruciform or regular structure being kinetically

† The following values were used in this calculation: NK/RT, 1,050 (ref. 19); helical pitch, 10.5 base pairs per turn; linking difference of pUC7 T_{12} (corrected to 50°C), -11.0.

trapped in that state for relatively long times.

Slow rates of cruciform extrusion and reabsorption have also recently been reported by other workers (25).

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